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CONTENTS OF THIS ISSUE

The Synthesis and Antifungal Properties of 6-Fluorothymol 689
C. A. Discher, J. M. Cross, P. F. Smith, and *M. Iannarone*
The Effect of pH on the Efficiency of Various Mold Inhibiting Compounds 691
F. J. Bandelin
Tragacanth Solutions III. The Effect of pH on the Stability 695
T. W. Schwarz, Gerhard Levy, and *H. H. Kawagoe*
A Mathematical Interpretation of the Kinetics of Elevated Temperature Stability Studies as Applied to Solid Formulations 697
Stuart P. Eriksen, John F. Pauls, and *Joseph V. Swintosky*
An Inverse Isotope Dilution Analysis of Salicylic Acid 701
H. A. Swarin and *J. E. Christian*
The Effect of Zinc Acetate on Red Corpuscles 703
Donald E. Cadwallader, Jr. and *William J. Husa*
Isotonic Solutions VI. The Permeability of Red Corpuscles to Various Salts of Organic Acids 705
Donald E. Cadwallader, Jr. and *William J. Husa*
Isotonic Solutions VII. The Permeability of Red Corpuscles to Various Alkaloidal Salts 711
C. Larry Thomasson and *William J. Husa*
Fluorescence of Powdered Vegetable Drugs Under Ultraviolet Radiation 715
Charles J. Kokoski, Robert J. Kokoski, and *Frank J. Slama*
The Pharmacology of Some Bis-Quaternary Ammonium Compounds 718
Zola P. Horovitz, Edward C. Reif, and *Joseph P. Buckley*
The Culture of *Claviceps purpurea* IV. Lipogenesis 721
James E. Dusenberry and *Arthur E. Schwarting*
Degradation of Epinephrine Induced by Bisulfite 723
Louis C. Schroeter, Takeru Higuchi, and *Edward R. Schuler*
Colorimetric Estimation of Some Cholinergic Esters. Application to the Demonstration of Acetylcholinesterase Activity 728
Jacob S. Hanker, Alan Gelberg, and *Benjamin Witten*
The Sterol and Triterpene Content of the Labiatae Family 731
Harold J. Nicholas
Dialkylaminoalkyl Esters and Amides Derived from 3-Hydroxy- and 3-Alkoxy-2-naphthoic Acids 734
George M. Sieger, William M. Ziegler, David X. Klein, and *Herman Sokol*
The Pharmacologic Actions of the Bronchodilator Drug, Isoetharine 744
A. M. Lands, F. P. Luduena, J. O. Hoppe, and *Irene H. Oyen*
Examination of the Physical Chemical Basis for the Isoniazid-*p*-Aminosalicylic Acid Combination 749
Jerome A. Reinstein and *Takeru Higuchi*
Sulfaethylthiadiazole IV. Steady State Blood Concentration and Urinary Excretion Data Following Repeated Oral Doses 753
Joseph V. Swintosky, Amedeo Bondi, Jr., and *Manford J. Robinson*
A Note on the Extraction and Fluorescence of Some Oxidation Products of Reserpine 757
Earl B. Dechene
A Note on the Use of the Brookfield RVF Viscometer with Thickening Agent Dispersions 758
J. O. Runkis, Nathan A. Hall, and *J. Wait Rising*
A Note on the Photosensitivity of Phenothiazine Derivatives 760
Louis J. Ravin, Lloyd Kennon, and *Joseph V. Swintosky*
Book Notices 761
Advertising ..... Cover pages 2, 3, 4, and I to IV

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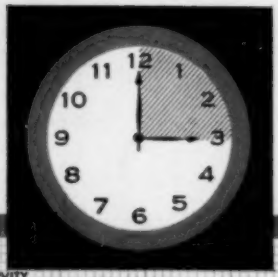

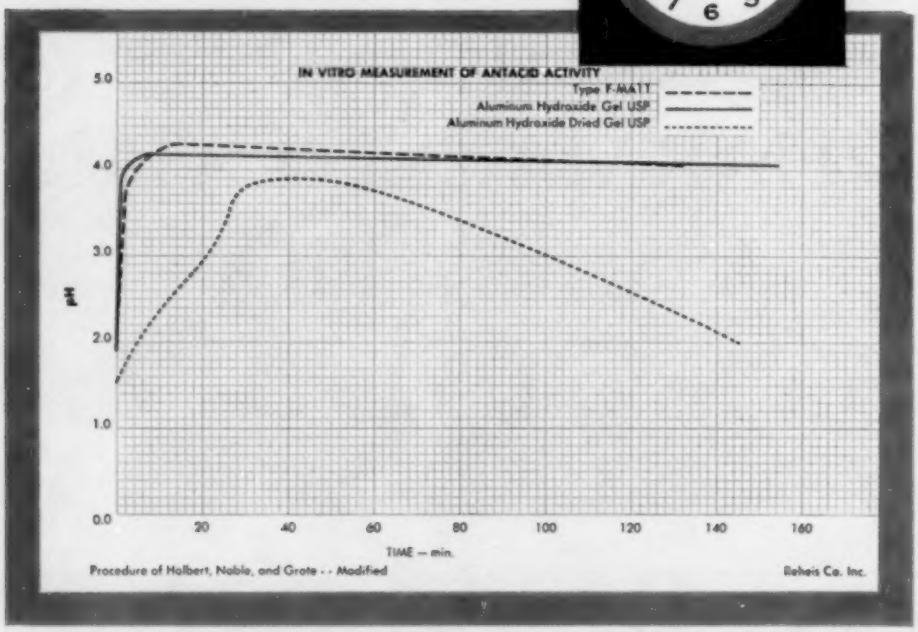

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1959 Iodine Research Award Nominations Requested Prior to January 1, 1959

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1959 Chilean Iodine Educational Bureau, Inc., Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine.

The award consists of $1,000 and a diploma setting forth the reasons for selection of the recipient. It may be presented each year at the annual meeting of the ASSOCIATION.

Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1959 Award, *nominations must be received on or before January 1, 1959.*

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During the period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Chilean Iodine Educational Bureau, Inc.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION's Council. The present committee includes Justin L. Powers, chairman; F. F. Blicke, E. A. Brecht, John E. Christian, Stanley G. Mittelstaedt, Lloyd M. Parks, and Linwood F. Tice.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. Travel expense to the meeting at which the award is to be made is provided for up to $250.

The award is now in its eleventh year. The recipient of the first award was Dr. William T. Salter, Yale University pharmacologist; the second recipient was Dr. George M. Curtis, Chairman of the Department of Surgical Research and Professor of Surgery at Ohio State University, and the third was Dr. C. P. Leblond, Professor of Anatomy, McGill University, Montreal, Canada. The fourth award was shared jointly by Dr. George Moore of the University of Minnesota Medical School and Dr. Moses Ashkenazy, Chief of Neurosurgery, Veterans Hospital, Houston, Tex. Dr. Vernon H. Wallingford, Mallinckrodt Chemical Works, St. Louis, Missouri, was the recipient of the fifth award. Dr. Jack Gross, Associate Professor of Anatomy, State University of New York, College of Medicine in Brooklyn, received the sixth award and Dr. Dominick Papa, Schering Corporation, Bloomfield, N. J., received the seventh award. The eighth award went to Dr. John E. Christian, Professor of Pharmaceutical Chemistry, School of Pharmacy, Purdue University, Lafayette, Indiana. The ninth award was conferred on Dr. James O. Hoppe, Research Pharmacologist, Sterling-Winthrop Research Institute, Rensselaer, New York, during the 1957 Convention of the AMERICAN PHARMACEUTICAL ASSOCIATION. No award was made in 1958.

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The next annual meeting of the American Pharmaceutical Association will be held in Cincinnati, Ohio, April 19 to 24, 1959. Titles and abstracts (not to exceed 200 words) of papers must be submitted to the appropriate Section secretary not later than February 1, 1959, or preferably earlier, in order to assure a place on any Section program.

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The Synthesis and Antifungal Properties of 6-Fluorothymol*

By C. A. DISCHER, J. M. CROSS, P. F. SMITH†, and M. IANNARONE

The compound 6-fluorothymol was synthesized and its antifungal activity was compared with those of thymol and other 6-halothymols by *in vitro* test. Nine organisms, including six species of trichophyton, two species of microsporum, *Candida albicans*, and *Staphylococcus aureus*, were used in these tests. The results indicate that at a concentration of 0.05 per cent in propylene glycol solution, 6-iodothymol was the most effective agent, followed by 6-bromothymol, 6-chlorothymol, 6-fluorothymol, and thymol.

IN A PREVIOUS PAPER (1), the synthesis and antifungal properties of thymol, 6-chlorothymol, 6-bromothymol, and 6-iodothymol were presented. Because of its unavailability, 6-fluorothymol was not included at that time. Dunker (2) reported a compound, melting at 57°, prepared by the diazonium reaction, but failed to establish its identity. Hach (3) prepared 6-fluorothymol by diazotization of 6-aminothymol methyl ether, decomposition of the resulting diazonium fluoroborate by heating to 150°, and hydrolysis of the resulting 6-fluoro ether. He reported a melting point of 52°.

EXPERIMENTAL

Synthesis of 6-Fluorothymol.—6-Aminothymol was prepared (4) as the fluoroborate salt and diazotized in the usual manner. The thick slurry obtained was filtered as well as possible with suction, and the resulting cake was further dried by pressing in filter paper. This damp product was then dissolved in ethyl acetate and the solution dried with anhydrous sodium sulfate. The dry solution was filtered, and the residue extracted several times with dry ethyl acetate. The combined filtrates were set aside to evaporate at room temperature. The diazonium salt crystallized from this solution as long needles. It was purified by extracting the mass of crystals with successive portions of dry petroleum ether until the extract became practically colorless. The crystals were dried and the extraction process repeated using dry ether. The crystals prepared in this way varied from colorless to a very light tan. On the melting point block, it was found that decomposition began in the range, 75–80°. The yield of diazonium salt was about 83% of theory.

A 250-ml. portion of an approximately 5:1 mixture of benzene and toluene was placed in a 500-ml. flask with 10.0 Gm. of the diazonium salt. The salt was maintained in suspension by vigorous stirring and the mixture heated sufficiently to allow the suspending solvent, together with the by-product gases, to distill slowly. The resulting condensate showed no 6-fluorothymol. When the reaction was complete, as indicated by the formation of a clear solution from the suspension, the reaction mixture was allowed to cool and was filtered. The solution was placed in a crystallizing dish and the organic solvent allowed to evaporate at room temperature. The resulting thick slurry was treated with sufficient 6% NaOH to dissolve the 6-fluorothymol and then diluted to about ten times its volume with water and filtered. The residue was washed several times with water and discarded. The combined extract and water washings were cooled with crushed ice and slowly brought to a pH of about 7 by addition of concentrated HCl. Ice was added when necessary so that some was pres-

* Received April 25, 1958, from the Research Laboratories of Rutgers University, College of Pharmacy, Newark 4, N. J.

Presented to the Scientific Section, A. PH. A., Los Angeles meeting, April, 1958.

† Present address: College of Pharmacy, University of Rhode Island, Kingston.

ent at all times. The compound tended to separate as an oil if the solution was not kept cold during neutralization. The compound separated from this mixture, especially with vigorous stirring, as pale yellow crystals. The yield was 70% of theory.

The 6-fluorothymol was purified by vacuum sublimation at a temperature sufficient to melt the crude compound, and by using a water aspirator as the reduced pressure source. The 6-fluorothymol sublimed as clear, colorless crystals. The colored impurity was not volatile under these conditions, except at the very end of a run.

It was found that as the product was purified by repeated sublimation, a melting point of 55.6–56°, was obtained. The less pure samples melted at slightly lower temperatures.

Anal.—Calcd. for $C_{10}H_{13}FO$: C, 71.40; H, 7.79; F, 11.30. Found: C, 71.30; H, 7.82; F, 11.19.

Antifungal Activity.—In order to evaluate the antifungal properties of the 6-halothymols, *in vitro* studies were performed to compare their effectiveness against a number of dermatophytes chiefly of the genera Trichophyton and Microsporon, and against *Candida albicans*. Since there is such a wide variation in the testing of these compounds *in vitro*, the method selected was that of the FDA, serum agar cup plate method as described in Circular 198 (5). Since serum agar cup plates do not differentiate between fungistatic and fungicidal action, fungistatic action was determined by the standard technique of measuring surface and subsurface zones of inhibition.

A preliminary investigation indicated that all of the thymol compounds were active in concentrations of 5% or more. It was in the range of 0.05% to 1.0% that significant differences in activity were noticed. Consequently, this latter range was used for purposes of comparison. Table I demonstrates

TABLE I.—INHIBITION, DIAM. IN MM., AGAINST EIGHT FUNGAL ORGANISMS BY FLUOROTHYMOL[a]

Organism	Percentage Fluorothymol 1.0 S[c]	1.0 SS[c]	0.1 S	0.1 SS	0.05 S	0.05 SS	Sterile P.G.[b] 100%
C. albicans	8.00	8.00	4.0	3.0	4.0	3.0	0.0
M. felineum	8.00	8.00	2.0	2.0	1.0	0.0	0.0
M. canis (Stilliansi)	15.0	15.0	1.0	1.0	1.0	0.0	0.0
T. purpureum (Emmons 661)	18.0	18.0	4.0	4.0	1.0	0.0	0.0
T. interdigitale	17.0	17.0	N.R.	N.R.	1.0	0.0	0.0
T. interdigitale (Emmons 640)	18.0	18.0	5.0	4.0	2.0	0.0	0.0
T. interdigitale 598B	16.0	16.0	4.0	4.0	2.0	0.0	0.0
T. gypseum	16.0	16.0	1.0	1.0	1.0	0.0	0.0

[a] Each result is an average of three plates for each percentage tested in the presence of 10% horse serum and after fourteen days incubation.
[b] Sterile propylene glycol was used as a control and solvent for the fluorothymol.
[c] S = Surface, SS = Subsurface inhibition.

TABLE II.—INHIBITION, DIAM. IN MM., AGAINST THREE FUNGAL ORGANISMS BY 6-HALOTHYMOLS[a]

Thymol Compound	%	Organism 1[b] S[e]	Organism 1[b] SS[e]	Organism 2[c] S	Organism 2[c] SS	Organism 3[d] S	Organism 3[d] SS
6-Iodothymol	0.05	4.00	4.00	11.00	11.00	5.00	5.00
6-Bromothymol	0.05	4.00	4.00	8.00	8.00	2.00	2.00
6-Chlorothymol	0.05	0.00	0.00	N.R.[f]	N.R.	0.00	0.00
6-Fluorothymol	0.05	1.00	0.00	1.00	0.00	4.00	2.00
Thymol	0.05	0.00	0.00	0.00	0.00	0.00	0.00
Propylene glycol[g]	100.00	0.00	0.00	0.00	0.00	0.00	0.00

[a] Each result is an average of three plates for each percentage tested after fourteen days incubation.
[b] *Trichophyton gypseum*.
[c] *Trichophyton interdigitale*.
[d] *Candida albicans*.
[e] S = Surface, SS = Subsurface.
[f] N.R. = No results.
[g] Sterile propylene glycol was used as a control and solvent for all the halothymols.

TABLE III.—INHIBITION, DIAM. IN MM., AGAINST THREE FUNGAL ORGANISMS BY 6-HALOTHYMOLS[a]

Thymol Compound	%	Organism 1[b] S[e]	Organism 1[b] SS[e]	Organism 2[c] S	Organism 2[c] SS	Organism 3[d] S	Organism 3[d] SS
6-Iodothymol	0.05	8.00	8.00	6.00	6.00	8.00	8.00
6-Bromothymol	0.05	0.00	0.00	9.00	3.00	5.00	5.00
6-Chlorothymol	0.05	1.00	1.00	4.00	4.00	2.00	2.00
6-Fluorothymol	0.05	1.00	0.00	2.00	0.00	2.00	0.00
Thymol	0.05	0.00	0.00	0.00	0.00	0.00	0.00
Propylene glycol[f]	100.00	0.00	0.00	0.00	0.00	0.00	0.00

[a] Each result is an average of three plates for each percentage tested after fourteen days incubation.
[b] *Trichophyton purpureum* (Emmons 661).
[c] *Trichophyton interdigitale* (Emmons 640).
[d] *Trichophyton interdigitale* (598B).
[e] S = Surface, SS = Subsurface.
[f] Sterile propylene glycol was used as a control and solvent for all the halothymols.

TABLE IV.—INHIBITION, DIAM. IN MM., AGAINST TWO FUNGAL ORGANISMS BY 6-HALOTHYMOLS[a]

Thymol Compound	%	Organism 1[b] S[d]	Organism 1[b] SS[d]	Organism 2[c] S	Organism 2[c] SS
6-Iodothymol	0.05	5.00	5.00	5.00	5.00
6-Bromothymol	0.05	8.00	8.00	8.00	8.00
6-Chlorothymol	0.05	8.00	8.00	5.00	5.00
6-Fluorothymol	0.05	1.00	0.00	1.00	0.00
Thymol	0.05	0.00	0.00	0.00	0.00
Propylene glycol[e]	100.00	0.00	0.00	0.00	0.00

[a] Each result is an average of three plates for each percentage tested after fourteen days incubation.
[b] *Microsporum felineum.*
[c] *Microsporum canis.*
[d] S = Surface, SS = Subsurface.
[e] Sterile propylene glycol was used as a control and solvent for all the halothymols.

the results of certain percentages of 6-fluorothymol in propylene glycol against eight fungal organisms. Tables II, III, and IV summarize the results of antifungal activity of thymol, 6-chlorothymol, 6-bromothymol, 6-iodothymol, and 6-fluorothymol against eight organisms. Table V summarizes the results of antibacterial activity of the same compounds. On the basis of the data shown, it is apparent that 6-fluorothymol ranks below the other halothymols in antifungal activity, according to the procedure used.

TABLE V.—INHIBITION, DIAM. IN MM., AGAINST *Staphylococcus Aureus* (*209*) BY 6-HALOTHYMOLS[a]

Thymol Compounds	Percentages 1.0 S[b]	1.0 SS[b]	0.1 S	0.1 SS	0.05 S	0.05 SS
6-Iodothymol	13.0	8.0	5.0	5.0	5.0	5.0
6-Chlorothymol	11.0	7.0	5.0	5.0	5.0	5.0
6-Bromothymol	12.0	7.0	4.0	4.0	3.0	3.0
6-Fluorothymol	10.0	10.0	3.0	3.0	3.0	2.0
Thymol	5.0	5.0	0.0	0.0	0.0	0.0
Phenol Control (5%)	4.0	4.0	4.0	4.0	4.0	4.0
Propylene glycol (100%)[c]	0.0	0.0	0.0	0.0	0.0	0.0

[a] Each result is an average of two plates for each percentage tested after forty-eight hours incubation.
[b] S = Surface, SS = Subsurface.
[c] Sterile propylene glycol was used as control and solvent for all the halothymols.

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The Effect of pH on the Efficiency of Various Mold Inhibiting Compounds*

By F. J. BANDELIN†

Twelve compounds having antifungal activity were investigated at pH levels of 3, 5, 7, and 9 for the effects of pH changes on their relative efficacy against four common mold contaminants. Tests conducted utilized standardized spore suspensions planted on sterile slants of a modified Sabouraud's agar medium containing graded amounts of the antifungal compounds. A majority of the compounds investigated showed a progressive loss of antifungal activity with increasing pH values to the point that some were totally ineffective in the neutral and alkaline range.

NUMEROUS STUDIES concerning the efficiency of various antifungal and preservative agents have appeared in the pharmaceutical literature and recommendations for effective concentrations of these compounds have been given for the preservation of pharmaceutical and cosmetic preparations. Much of this information is based on empirical work for, although some knowledge of the influence of pH on activity has been gathered for some of the preservatives available, no systematic or comprehensive investigation of the effect of pH on the efficiency of various antifungal compounds has appeared.

* Received November 20, 1957, from Flint, Eaton and Co., Decatur, Ill.
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Probably the oldest and most widely used preservatives in the food and drug and cosmetic industries are benzoic and salicylic acids. Their activity in acid media is well known as is their lack of activity in neutral or alkaline media. The history and use of these compounds is adequately reviewed by McCulloch (1) Gershenfeld (2), and others (3).

The alkyl esters of *p*-hydroxybenzoic acid are also widely used in the pharmaceutical industry. The history of their practical application dates back to 1924 when Sabalitschka (4, 5) presented the first of his numerous reports on their antibacterial and antifungal activity. His prodigious investigations with the esters of *p*-hydroxybenzoic acid resulted in his more than 60 pub-

lications on the subject. His work in this field has been reviewed by Neidig and Burrell (6), and by Bandelin (7). More recent work on these compounds, including acute and chronic toxicity, and the physiological disposition of these compounds has been published by Aalto, *et al.* (8), Matthews, *et al.* (9), and by Jones, *et al.* (10).

Kojic acid is of interest because it is one of the first antibiotic substances isolated. It is produced by Aspergilli and was first isolated in 1907 by Saito (11). Its activity as an antibacterial and antifungal agent was first reported by Yabuta in 1912, (12, 13). It is, however, not a particularly potent antifungal compound.

In 1913, Kiesel (14) published an extensive study of the relationship between chemical structure and fungicidal activity in the fatty acid series. Interest was not aroused however until Peck and his associates (1938, *et seq.*) reinvestigated the fungicidal properties of fatty acids and demonstrated that the antifungal activity of sweat was due to the fatty acids contained therein, and introduced sodium propionate as a clinical fungicide (15–17). Sodium propionate has become widely used as a preservative in the food industry, especially in bread and cheese (18, 19).

Dehydroacetic acid, although not widely used, is potentially an excellent preservative. Chemically it is 3-acetyl-6-methyl-1,2-pyran-2,4(3H)-dione. It is prepared by the interaction of two moles of ethyl acetoacetate (20). The application of dehydroacetic acid as a preservative for food and dairy products has been investigated and reported (21–23). It is active against both bacteria and fungi (24).

Sorbic acid is a development of the work of Gooding (25) who demonstrated that certain of the α,β-unsaturated fatty acids are suitable fungistatic agents for foods. It is synthesized from crotonaldehyde and malonic acid (26). Smith and Rollin (27) found it superior to sodium benzoate as a fungistatic agent for cheese products and Puls, *et al.* (28), have investigated its use as a preservative for pharmaceutical preparations.

Both vanillin and ethyl vanillin have found use both as food preservatives (29, 30) and in therapy (31, 32).

In all the literature pertaining to these various compounds, actual data concerning the specific effect of relative acidity or alkalinity on their activity is exceedingly sparse. We have selected these compounds, some established preservatives, and some new, in order to compare their activities at various pH levels over a broad range against four common molds which are likely contaminating agents. We have attempted to determine which of the compounds offer the best activity at a given pH in an effort to assist in selections for pharmaceutical formulations. The investigations carried on in this laboratory are therefore reported here.

EXPERIMENTAL

The test cultures employed in this work were *Aspergillus niger*, *Penicillium citrinum*, *Alternaria solani*, and *Chaetomium globosum*. These were propagated and spores derived by inoculating the surface of a Sabouraud's agar medium, poured to a depth of approximately 1/4 inch, in the bottom of a 250-ml. Erlenmeyer flask, with spores of the test organism and incubating at 30°, for twenty-one days. At the end of this time the spores were harvested by washing the surface of the medium with sterile distilled water, using aseptic technique, and diluting the washings with sterile water to a standard suspension containing 20,000 spores per ml. as determined by serial dilution and counting on agar plates.

The modified Sabouraud's agar employed was enriched with yeast extract and consisted of the following:

Peptone	10 Gm.
Agar	18 Gm.
Corn syrup solids[1]	40 Gm.
Yeast extract (Difco)	2 Gm.
Buffer solution, *q. s.*	1,000 ml.

Buffer solutions were prepared at pH 3, 5, 7, and 9 by mixing 0.2 *M* dibasic sodium phosphate solution with 0.1 *M* citric acid solution. These citrate-phosphate buffers were used to prepare the agar media which were readjusted with a pH meter at 45° in the fluid state after the addition of the test compounds.

The test compounds employed were as previously mentioned; benzoic acid, salicylic acid, the methyl, ethyl, propyl, and butyl esters of *p*-hydroxybenzoic acid, kojic acid, propionic acid, dehydroacetic acid, sorbic acid, vanillin, and ethyl vanillin.

Where necessary to put these compounds into solution their sodium salts were used. They were dissolved in the warm, fluid agar medium at each designated pH level in concentrations of from 0.2% in decreasing increments to the lowest concentration required to exhibit inhibition (0.001%) as indicated in the following tables. Slants were prepared at each pH and concentration level using 8 ml. of media per tube and these were plugged with cotton and sterilized by autoclaving.[2]

Five slants were prepared at each concentration and for each compound for each of the four test organisms. Slants were inoculated with one drop of standard spores suspension placed on the surface of the slant. All tubes were incubated at 30° and observed daily for fourteen days. Reproducibility was

[1] Used instead of dextrose.

[2] At pH 9.0, the esters of *para*-hydroxybenzoic acid, sorbic acid, vanillin, and ethyl vanillin were added to 7 cc. of the agar medium concentrate, at 50°, using 1 cc. sterile solutions of their sodium salts, sterilized by filtration, and of appropriate concentration to produce the desired concentration in the completed media. This was done to avoid hydrolytic destruction on autoclaving. This produced no noticeable change in pH.

good in all cases, and the criterion of inhibition was lack of visible growth in all five tubes at the end of the fourteen-day incubation period. Where growth occurred in all tubes at the 0.2% level it is indicated by a plus sign since this concentration was the maximum used for all compounds. Otherwise the lowest concentration at which no growth occurred in fourteen days is indicated in the following tables.

DISCUSSION

It is apparent from the data in these tables that the majority of the compounds, while fairly active antifungal agents at pH values of 3.0 and 5.0, became progressively less active as the pH increased so that at the higher pH levels of 7.0 and 9.0 they were relatively ineffective or only slightly active against the molds.

Kojic acid, the least active of the group, exhibited slight activity barely within the upper limit of concentration of the test procedure. At 0.2 per cent concentration it did not inhibit *Aspergillus niger*, even at pH 3.0.

Benzoic and salicylic acids were active inhibitors at pH 3.0 and 5.0; salicylic acid being more effective in lower concentrations than benzoic acid. At pH 7.0 and 9.0 however, no activity was evident at the maximum concentration of 0.2% in the media.

The esters of *p*-hydroxybenzoic acid were least affected by pH changes. There was a slight reduction in activity at the higher pH levels, but nothing like the reduction of activity encountered with most of the other compounds in the alkaline range. It is a well-known fact that the activity of these esters increases with increasing molecular weight. In this respect, an interesting observation was made during the investigation concerning the apparent progressive loss of differential ratio in activity of the higher molecular weight esters with increasing pH

TABLE I.—MINIMUM CONCENTRATION (PER CENT IN MEDIA) REQUIRED FOR INHIBITION OF MOLD GROWTH AT pH 3.0

	Chaetomium globosum	*Alternaria solani*	*Penicillium citrinum*	*Aspergillus niger*
Kojic acid	0.08	0.20	0.20	+
Benzoic acid	0.08	0.10	0.10	0.04
Salicylic acid	0.02	0.02	0.02	0.04
Methyl *p*-hydroxybenzoate	0.06	0.06	0.05	0.08
Ethyl *p*-hydroxybenzoate	0.02	0.02	0.02	0.04
Propyl *p*-hydroxybenzoate	0.008	0.015	0.005	0.02
Butyl *p*-hydroxybenzoate	0.003	0.005	0.002	0.015
Propionic acid	0.04	0.04	0.04	0.08
Dehydroacetic acid	0.001	0.001	0.001	0.002
Sorbic acid	0.01	0.005	0.02	0.04
Vanillin	0.08	0.02	0.06	0.10
Ethyl vanillin	0.04	0.01	0.02	0.06

TABLE II.—MINIMUM CONCENTRATION (PER CENT IN MEDIA) REQUIRED FOR INHIBITION OF MOLD GROWTH AT pH 5.0

	Chaetomium globosum	*Alternaria solani*	*Penicillium citrinum*	*Aspergillus niger*
Kojic acid	+	+	+	+
Benzoic acid	0.10	0.15	0.20	0.20
Salicylic acid	0.10	0.20	0.10	0.15
Methyl *p*-hydroxybenzoate	0.06	0.08	0.08	0.10
Ethyl *p*-hydroxybenzoate	0.03	0.04	0.03	0.06
Propyl *p*-hydroxybenzoate	0.01	0.02	0.01	0.03
Butyl *p*-hydroxybenzoate	0.008	0.01	0.005	0.02
Propionic acid	0.04	0.06	0.08	0.08
Dehydroacetic acid	0.005	0.002	0.005	0.005
Sorbic acid	0.06	0.02	0.08	0.08
Vanillin	0.01	0.06	0.10	0.20
Ethyl vanillin	0.06	0.04	0.04	0.06

TABLE III.—MINIMUM CONCENTRATION (PER CENT IN MEDIA) REQUIRED FOR INHIBITION OF MOLD GROWTH AT pH 7.0

	Chaetomium globosum	*Alternaria solani*	*Penicillium citrinum*	*Aspergillus niger*
Kojic acid	+	+	+	+
Benzoic acid	+	+	+	+
Salicylic acid	+	+	+	+
Methyl *p*-hydroxybenzoate	0.08	0.08	0.08	0.10
Ethyl *p*-hydroxybenzoate	0.04	0.05	0.04	0.06
Propyl *p*-hydroxybenzoate	0.02	0.03	0.02	0.04
Butyl *p*-hydroxybenzoate	0.01	0.02	0.015	0.02
Propionic acid	0.15	+	+	0.20
Dehydroacetic acid	0.04	0.02	0.04	0.04
Sorbic acid	0.10	0.08	0.08	0.10
Vanillin	0.10	0.10	0.10	0.20
Ethyl vanillin	0.06	0.04	0.04	0.06

TABLE IV.—MINIMUM CONCENTRATION (PER CENT IN MEDIA) REQUIRED FOR INHIBITION OF MOLD GROWTH AT pH 9.0

	Chaetomium globosum	*Alternaria solani*	*Penicillium citrinum*	*Aspergillus niger*
Kojic acid	+	+	+	+
Benzoic acid	+	+	+	+
Salicylic acid	+	+	+	+
Methyl *p*-hydroxybenzoate	0.10	0.10	0.15	0.15
Ethyl *p*-hydroxybenzoate	0.06	0.08	0.10	0.08
Propyl *p*-hydroxybenzoate	0.04	0.05	0.06	0.05
Butyl *p*-hydroxybenzoate	0.02	0.03	0.03	0.03
Propionic acid	+	+	+	+
Dehydroacetic acid	0.10	0.20	0.20	0.20
Sorbic acid	+	+	0.20	+
Vanillin	0.10	0.10	0.10	+
Ethyl vanillin	0.06	0.04	0.04	0.06

values. Whereas the differential between the methyl and butyl *p*-hydroxybenzoates with *Chaetomium globosum* at pH 3.0 is a ratio of 20:1, at pH 9.0 the differential is 5:1. Against *Alternaria solani* at pH 3.0 the ratio is 12:1, at pH 9.0 it is 3.3:1. Against *Penicillium citrinum* at pH 3.0 the differential ratio is 25:1, while at pH 9.0 it is 5:1; while against *Aspergillus niger* at pH 3.0 it is 10:1, at pH 9.0 it becomes 5:1.

The activity of propionic acid which was quite high at pH 3.0 was fair at pH 5.0 and was considerably reduced at pH 7.0. It failed completely at pH 9.0.

Dehydroacetic acid exhibited excellent activity at pH 3.0 and 5.0, and good activity at pH 7.0, but had rather poor activity at pH 9.0.

Sorbic acid was similar but superior to propionic acid in activity. Also like propionic acid it had no activity at the 0.2% level at pH 9.0.

Both vanillin and ethyl vanillin had good activity at pH 3.0, and although their activity was slightly reduced at pH 5.0, it seemed to be relatively little affected by pH changes above 5.0. At pH 9.0 both exhibited activity with ethyl vanillin being comparable to the esters of *p*-hydroxybenzoic acid.

Although much of the recent work on fungicides has been done utilizing the agar cup plate or filter paper disk techniques, we felt that although these methods were satisfactory for comparing the relative efficiency of these compounds with each other, the technique employed here was more suited to practical application in that it gives an idea of the actual concentration of the compounds required to produce complete inhibition of the molds at a given pH in a highly nutrient medium. That this modification of Sabouraud's agar enriched with yeast extract is a highly acceptable medium for growth of the organisms was illustrated by the fact that the organisms grew luxuriantly upon it at all pH levels in control tubes containing no inhibitor.

SUMMARY AND CONCLUSIONS

Twelve compounds having antifungal activity were investigated for their effect against four common molds at pH levels of 3, 5, 7, and 9. The molds used were *Chaetomium globosum*, *Alternaria solani*, *Penicillium citrinum*, and *Aspergillus niger*. Spores of these organisms were inoculated upon the surface of a modified Sabouraud's agar enriched with yeast extract and containing varying concentrations of the preservative compounds up to a maximum of 0.2 per cent. Criterion of inhibition was lack of visible growth in all of five tubes at any concentration after incubation at 30° for fourteen days.

The organic acids such as benzoic, salicylic, propionic, and sorbic all lost activity with increasing pH. Kojic acid was the poorest inhibitor at all levels. Dehydroacetic acid had excellent activity in the acid range, but its activity was also reduced at the alkaline level.

The activity of the esters of *p*-hydroxybenzoic acid was only slightly affected by pH changes and these compounds probably have the greatest utility as preservatives for pharmaceutical preparations generally. Progressive loss of differential in activity was noted with the higher molecular weight esters with increasing pH values.

Vanillin and ethyl vanillin appear to have marked preservative properties which are not materially affected by pH changes. Their activity shows little change even at elevated pH levels.

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Tragacanth Solutions III.*

The Effect of pH on the Stability

By T. W. SCHWARZ, GERHARD LEVY,† and H. H. KAWAGOE

The effect of pH on the viscosity of tragacanth solutions during storage was studied. Tragacanth solutions exhibited maximum stability near pH 5.

THE USE of tragacanth as a thickening and suspending agent for jellies, emulsions, and suspensions of various pH makes an investigation of the effect of pH on the stability of the gum of interest.

According to Walter (1), tragacanth has poor alkali stability. Becker (2) stated that tragacanth appeared more stable in dilute acid solutions than in alkaline solutions, while Mantell (3) declared that the viscosity of tragacanth mucilages was reduced by adding acid or alkali.

A frequently cited paper by Schon and Fuerst (4) reported that the maximum viscosity of tragacanth solutions occurs at pH 8. However, their work determined the immediate effect of pH upon the viscosity but did not include extended storage tests.

We have studied the effect of pH on the stability by subjecting tragacanth solutions, which were buffered at various pH, to accelerated storage tests. Since the most frequently desired property of the gum is its thickening capacity, we have taken the rate of the viscosity decrease of the solutions as the measure of degradation.

In a previous paper (5), it was pointed out that the effect of additives on the rate of degradation of tragacanth should be studied on maximally hydrated gum, rather than on the partially hydrated gum usually obtained when tragacanth is dissolved in cold water by agitation. A method for approaching complete hydration by repeated homogenizations was described in the paper. In solutions where the gum is only partially hydrated, the additives may also affect the rate of hydration and therefore their effect on the stability will be more difficult to evaluate.

EXPERIMENTAL

Tragacanth Stock Solutions.—Stock solutions were prepared from tragacanth ribbon No. 1, U. S. P. Several different lots of gum, obtained from different sources, were used. The necessary amount of tragacanth ribbon was added to preserved water (methylparaben U. S. P. 0.1%, propylparaben U. S. P. 0.025% in distilled water), to which solutions of the additives were later added. The solution was agitated by a mechanical shaker for twenty-four hours. The resultant mucilage was forced six times through a hand homogenizer with constant nozzle adjustment to approach maximal hydration (5) and homogeneity. After the homogenizations, the material was again agitated by a mechanical shaker for one hour.

Buffer Stock Solutions.—Buffer solutions were prepared to contain 0.6*M* total buffer concentration. The following materials were used in the preparation of the solutions: Acetate buffers: acetic acid, glacial, reagent grade; sodium acetate, anhydrous, reagent grade. Phosphate buffers: monosodium phosphate, anhydrous, reagent grade; disodium phosphate, anhydrous, reagent grade. Maleate buffers: maleic acid, reagent grade; sodium hydroxide pellets, U. S. P.

Stock solutions of benzoic acid U. S. P., sodium chloride U. S. P., disodium phosphate, and monosodium phosphate were prepared by dissolving the substances in preserved water.

Preparation of Samples.—Seventy-five grams of tragacanth stock solution and 25 ml. of buffer stock solution (respectively a solution of a salt or an acid) were placed in four-ounce screw-capped jars. Controls consisted of 75 Gm. tragacanth stock solution and 25 ml. preserved water. The solutions were mixed by first shaking the jars vigorously by hand and then placing them on a mechanical shaker for one hour. The solutions were stored at room temperature for two days before the initial pH and viscosity were determined.

Viscosity Determination.—After vigorously shaking each jar, a sufficient quantity of material was placed in 50-ml. centrifuge tubes and the occluded air was removed by centrifugation. The tubes were then placed in a water bath adjusted to 25° ± 0.5° for two hours. Viscosity measurements were made with the Brookfield Synchro-Lectric Viscometer, Model LVF, spindle No. 4, at 6 r. p. m. Readings were taken after ten minutes of spindle rotation.

pH Determinations.—pH determinations were made with a Beckman pH meter, Model H-2.

Accelerated Aging.—The jars were stored in an electric oven at a temperature of 50° ± 1°.

RESULTS AND DISCUSSION

Each set of solutions was prepared from the same stock solution of tragacanth so that the degree of hydration and the concentration of the gum were the same in each sample. Various buffer systems were employed at a given pH in order to detect effects that may be specifically due to the components of the buf-

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fer rather than to pH. Thus it was found that citrates caused a rapid viscosity loss of tragacanth solutions regardless of pH; this is probably due to their complexing with calcium present in tragacanth.

Figures 1 and 2 show the effect of pH on the viscosity of solutions of two different lots of tragacanth. The same relationship was observed with solutions prepared from several other lots of tragacanth. The graphs show that the gum is most stable at a pH slightly above 5 and that the viscosity decreases markedly during storage when the pH differs by more than one-half unit from the optimum. The initial viscosity of all solutions, except those with alkaline pH, was uniform. At a pH of 8, the initial viscosity of tragacanth solutions was considerably higher, as already observed by Schon and Fuerst (4). However, after as little as twenty-four hours of storage at 50° the viscosity of these solutions had dropped precipitously to $^1/_4$ of the initial value, while the controls and the solutions adjusted to pH 5 showed no viscosity loss.

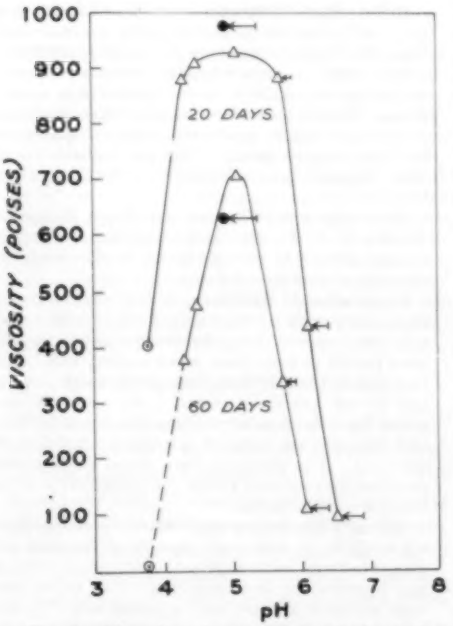

Fig. 1.—Viscosity of tragacanth solutions after twenty and sixty days' storage at 50°.

△ maleate buffer; ⊙ benzoic acid 0.2%; ● control.

Arrow designates drift from initial pH.

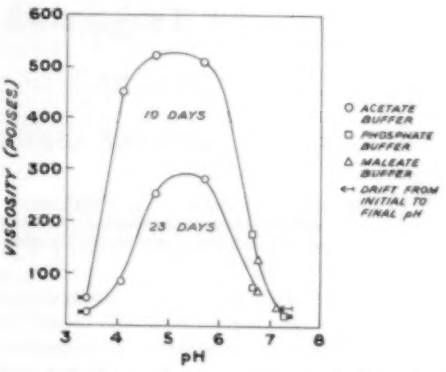

Fig. 2.—Viscosity of tragacanth solutions after ten and twenty-three days' storage at 50°.

The temporary viscosity maximum which was obtained at pH 8 is probably due to the solution of the alkali-soluble polysaccharide fraction, since tragacanth has been shown to be a mixture of a neutral, water-soluble polysaccharide and a complex, alkali-soluble acid polysaccharide (6). At more alkaline pH, the initial high viscosity obtained at pH 8 is not attained because of the rapid degradation of the gum in the more alkaline region. This is borne out by the observations of Byrum and Dale (7) who adjusted the pH of tragacanth solutions which had a normal pH of 6 to pH 7, 9, and 11 with sodium hydroxide. They found a maximum initial viscosity at pH 7 and a very much lower viscosity at pH 9.

Our investigation shows that the viscosity-stability of tragacanth solutions is greatly affected by the pH. At a pH below 4 or above 6, the stability of tragacanth is definitely decreased. For example, the low pH of solutions preserved with benzoic acid results in the rapid degradation of the gum (Fig. 1). At a pH close to 5, tragacanth solutions exhibit maximum stability.

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A Mathematical Interpretation of the Kinetics of Elevated Temperature Stability Studies as Applied to Solid Formulations*

By STUART P. ERIKSEN, JOHN F. PAULS, and JOSEPH V. SWINTOSKY

When using kinetic principles and the Arrhenius equation to predict shelf life, it is convenient to use exaggerated storage temperatures. However, it is sometimes difficult to attain temperature equilibrium rapidly in these cases, and because accurate heating time estimates are essential for precise shelf life predictions, the time lags during sample heating and cooling may seriously affect the prediction accuracy. In this paper an equation is presented which relates the sample heating rate, storage time, temperature, and the heat of activation of the breakdown reaction. The solution of the equation is expressed as "Equilibrium Temperature Time Equivalent" or ETTE, and represents the effective time of storage at the oven temperature. The ETTE takes into account the finite time lag in attaining temperature equilibrium during the heating phase and during cooling after removal from the oven. A sample calculation is presented to indicate the magnitude of the error involved in using sample storage time which does not correct for the time necessary to attain temperature equilibrium. The ETTE permits the formulator to correct either storage times or his sampling times, thus making the error negligible.

WHEN FORMULATING a new drug product, it is often possible to obtain a quick estimate of its shelf life by employing the concepts of chemical kinetics, and especially the Arrhenius equation relating breakdown rate and temperature. In order to obtain the information needed for an analysis of this type in as short a time as possible, it is customary to use samples stored at high temperatures for varying periods of time, some of which may be quite short. These shorter periods of storage may be subject to considerable error, however, when either the bulk or the heat conductivity of the formulation under test prevents rapid temperature equilibration (1). There may be a considerable time lag until the sample reaches the equilibrium storage temperature after placement in the oven; and subsequently, when the sample is removed from the oven, it may be some time before it reaches room temperature. Due to the exponential character of both the sample heating and cooling, and also of the rate of drug breakdown with temperature, it is incorrect to assume that these errors cancel each other.

The magnitude of the error introduced by ignoring the time necessary for heating and cooling to equilibrium temperatures is related to *1* the coefficients describing the rates of heating and cooling of the sample, *2* the duration of storage, *3* the storage temperature, or more exactly the difference between oven storage temperature and the initial sample temperature, and *4* activation energy of the breakdown reaction.

An equation which relates these factors has been derived. Solutions to the equation are called "ETTE" or "Equilibrium Temperature Time Equivalents." These may be described as storage times which correct for the finite heating and cooling time encountered in the usual exaggerated temperature storage study.

The derivation and discussion of this equation are presented here; its application to special pharmaceutical systems will be described in a later report.

EQUATIONS

When a packaged drug product or formulation to be tested for heat stability or shelf life is placed in an oven, it has been found experimentally that the internal temperature of the sample rises in nearly an exponential fashion, described approximately by the expression

Rate of temperature change =

$$\frac{d(T_{eq} - T)}{dt} = -Z_h(T_{eq} - T) \quad \text{(Eq. 1)}$$

where T_{eq} and T are the equilibrium oven (fixed) and sample (variable) temperatures, respectively, t is the time and Z_h is a heating rate coefficient, primarily dependent upon the nature of the drug product, the product container, the heating medium, and the difference in temperature between the sample and the heat source. By integration of Eq. 1 the temperature at any time t can be found from the equation

$$T = T_{eq} - (T_{eq} - T_o)e^{-Z_h t} \quad \text{(Eq. 2)}$$

T_o is the initial sample temperature, and in practice will be room temperature. Because the cooling which occurs after removal from the oven also takes place exponentially, corresponding equations may be written for this phase. The sample (variable)

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temperature, T, at any time t is found to be

$$T = T_{eq}' + (T_o' - T_{eq}')e^{-Zct} \quad \text{(Eq. 3)}$$

where T_{eq}' and T_o' now represent equilibrium room (fixed) temperature and sample temperature prior to removal from the oven, respectively. In most cases, T_o' is identical with the oven temperature.

The actual effect of this slow heating and cooling on the breakdown of a test sample may not be immediately apparent, but can be shown by studying a hypothetical case. If one assumes that the test drug breaks down by a pseudo-first order reaction, then the rate at which the drug concentration decreases at any given temperature is

$$\text{Rate} = -dc/dt = k_1 c \quad \text{(Eq. 4)}$$

where c and t are concentration and time, respectively, and k_1 (the specific rate constant) has the form

$$k_1 = Ae^{-\Delta H/RT} \quad \text{(Eq. 5)}$$

The terms ΔH, R, and T are energy of activation, the gas constant, and the temperature, respectively, while A is a probability factor. When T changes with time in the fashion described in Eqs. 1–3, one can determine the specific rate constant during heating (k_{1h}) from the equation

$$k_{1h} = Ae^{-\Delta H/R(Teq-(Teq-To)e^{-Zht})} \quad \text{(Eq. 6)}$$

and conversely, for cooling,

$$k_{1c} = Ae^{-\Delta H/R(Teq' + (To' - Teq')e^{-Zct})} \quad \text{(Eq. 7)}$$

where all the terms have the previous definitions.

Rewriting Eq. 4, and substituting in it Eq. 6 for the heating case, we obtain the expression

$$\frac{d \ln c}{dt} = -Ae^{-\Delta H/R(Teq - (Teq - To)e - Zht)} \quad \text{(Eq. 8)}$$

while if we use Eqs. 4 and 7 for the cooling case, we obtain

$$\frac{d \ln c}{dt} = -Ae^{-\Delta H/R(Teq' + (To' - Toq')e - Zct)} \quad \text{(Eq. 9)}$$

When integrated, these equations should yield a curve like the solid line in Fig. 1 when used to graph ln c *versus* t. Equation 8 would best describe the curve from t_0 to t_1, while Eq. 9 would describe the line from t_2 to t_3. From t_1 to t_2 Eq. 4 would apply. The curve thus obtained may be compared with a hypothetical curve postulated for the same drug but assuming instantaneous heating and cooling (broken line Fig. 1).

The symbol t_0 represents the moment of placement in the oven, t_1 the time when the sample has reached oven temperature, t_2 the time when the sample is removed from the oven (or oven time), and t_3 the time when the sample has reached room temperature again. After t_1 minutes the actual case (solid line) would have broken down an amount represented by AC^*, while in the same time the hypothetical case at constant temperature (broken line) would show a loss of AB. A hypothetical sample at oven temperature at t_0 would require only t_3 minutes to show the same loss (represented by

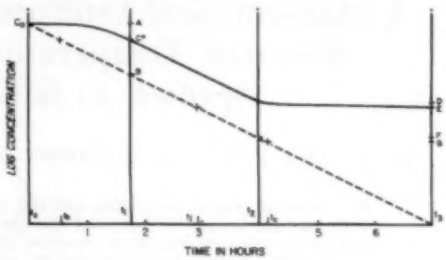

Fig. 1.—Showing the relationship between the hypothetical (broken line) and the actual (solid line) breakdown rates.

AC^*) as the actual case after t_1 minutes. After sample removal from the oven at t_2 similar reasoning relates quantities DE, FG and $(t_c - t_3)$. In $(t_3 - t_2)$ minutes the actual case has suffered a breakdown represented by DE, while the hypothetical case would have broken down this same amount (represented by FG) in $(t_c - t_2)$ minutes. Between t_1 and t_2 the temperature is constant, so that the rate of breakdown of the actual case is the same as the hypothetical.

Apparently then, the breakdown of the actual sample, at a changing temperature, in t_3 minutes is equivalent to the breakdown of the hypothetical case (broken line) which heats and cools instantaneously, in t_j minutes. The time t_j may be called the "Equilibrium Temperature Time Equivalent" or ETTE. This value can be calculated experimentally using the equation

$$\text{ETTE} = (\text{Oven time} - t_1) + \text{heating equivalent} + \text{cooling equivalent} = (t_2 - t_1) + t_h + (t_c - t_2) \quad \text{(Eq. 10)}$$

For the purpose of discussion it has been assumed that the hypothetical sample remained in the oven from t_2 to t_3, while the actual case was removed. This line is used only to determine the time t_c as described above. The hypothetical case, if removed from the oven at t_2, would instantly assume some nearly horizontal rate corresponding to the room temperature breakdown rate (a line parallel to the continuation of the actual case past t_2).

The ETTE may be calculated from kinetic principles with the following line of reasoning.

The reactant breakdown in the actual case (Fig. 1 solid line) after t_1 minutes is found by integration of Eq. 4

$$-\int_{\ln c_o}^{\ln c^*} d \ln c = \int_{t_o}^{t_1} k_{1h} dt = -(\ln c^* - \ln c_o) \quad \text{(Eq. 11)}$$

where c^* is the concentration after t_1 minutes.

The hypothetical sample at oven temperature (broken line) will break down according to a first order rate equation

$$-\frac{d \ln c}{dt} = k_{ov} \quad \text{(Eq. 12)}$$

where k_{ov} is the specific rate constant at equilibrium oven temperature, or by integration

$$-(\ln c - \ln c_o) = k_{ov} t \quad \text{(Eq. 13)}$$

Therefore the time required for the hypothetical case at constant temperature to break down any amount ($\ln c_o - \ln c$) may be found by calculation, using the relationship

$$t = \frac{\ln c_o - \ln c}{k_{ov}} \qquad \text{(Eq. 14)}$$

The amount of breakdown after t_1 minutes in the actual case (Eq. 11) may be substituted in Eq. 14, and in this case the time obtained from Eq. 14 is identical to t_h as defined in Fig. 1,

$$t_h = \frac{\ln c_o - \ln c^*}{k_{ov}} = \frac{\int_{t_0}^{t_1} k_{1h} dt}{k_{ov}} \qquad \text{(Eq. 15)}$$

Similar reasoning relates the amount of breakdown during the cooling period of the actual case to the time required for a theoretical sample maintained at oven temperature to achieve the same amount of breakdown, and the time obtained is ($t_c - t_2$),

$$(t_c - t_2) = \frac{\int_{t_2}^{t_3} k_{1c} dt}{k_{ov}} \qquad \text{(Eq. 16)}$$

where all terms have their previous definitions.

In order to determine the t_h, $t_c - t_2$ and ETTE it is only necessary to solve the following equations:

$$t_h = \frac{A \int_{t_0}^{t_1} e^{-\Delta H/R(T_{eq} - (T_{eq} - T_0)e^{-Z_h t})} dt}{k_{ov}} \qquad \text{(Eq. 17)}$$

$$(t_c - t_2) = \frac{A \int_{t_2}^{t_3} e^{-\Delta H/R(T_{eq'} + (T_0' - T_{eq'})e^{-Z_c t})} dt}{k_{ov}} \qquad \text{(Eq. 18)}$$

$$\text{ETTE} = (t_2 - t_1) + t_h + (t_c - t_2) \qquad \text{(Eq. 19)}$$

EVALUATION OF THE INTEGRAL

The integrals contained in the equations for the general case, Eqs. 17 and 18, are not definitely integrable, but by suitably selecting values for ΔH and A, values of Z_h and Z_c found by experiment for a stored sample may be used to calculate a solution numerically. The integral was solved using a Burroughs E 101 electronic computer, employing a slightly altered form of the equations. Using the substitution[1]

$$T = (T_{eq} - (T_o - T_{eq}) e^{-Z_h t}) \qquad \text{(Eq. 20)}$$

for the heating case, we find that

$$dt = \frac{dT}{Z_h(T_{eq} - T)} \qquad \text{(Eq. 21)}$$

Making this substitution and appropriately changing the limits, one obtains

$$t_h = \frac{A}{Z_h k_{ov}} \int_{RT}^{OT} \frac{e^{-\Delta H/RT} dT}{T_{eq} - T} \qquad \text{(Eq. 22)}$$

or for cooling

$$t_c = \frac{A}{Z_c k_{ov}} \int_{OT}^{RT} \frac{e^{-\Delta H\ RT} dT}{T_{eq} - T} \qquad \text{(Eq. 23)}$$

where RT and OT are room and oven temperature. The solution of the integral part then is independent of sample heating or cooling rate, and so is a characteristic only of the temperatures used and the reaction studied, and may be adjusted to any container size by simple division.

The complete equation can be simplified by making the substitutions

$$P_h = \frac{A}{k_{ov}} \int_{RT}^{OT} \frac{e^{-\Delta H/RT} dT}{T_{eq} - T} \qquad \text{(Eq. 24)}$$

$$P_c = \frac{A}{k_{ov}} \int_{OT}^{RT} \frac{e^{-\Delta H/RT} dT}{T_{eq} - T} \qquad \text{(Eq. 25)}$$

so that by determining P_h and P_c for a hypothetical reaction, the heating or cooling lag may be calculated for any sample container from the expressions

$$t_h = \frac{P_h}{Z_h} = \frac{P_h}{2.303 \times \text{Slope}} \qquad \text{(Eq. 26)}$$

$$t_c = \frac{P_c}{Z_c} = \frac{P_c}{2.303 \times \text{Slope}} \qquad \text{(Eq. 27)}$$

The slopes in these equations refer to the slopes as calculated from the semilog plots of sample temperature *versus* time (see Eq. 1), determined experimentally for each sample.

The solutions of P_h and P_c (Eqs. 24 and 25) for three temperatures have been tabulated in Table I using $\Delta H = 15$ Kcal and $A = 10^{10}$. When values for Z_h or Z_c are known or can be estimated, the ETTE can be calculated and used to estimate heating effect error as shown in the example.

Example.—The evaluation of ETTE for a hypothetical case. Assume the slope of the temperature *vs.* time plot for a 75° stored sample while heating is found to be 0.0313 and while cooling 0.0295, then

$$t_h = \frac{2.423}{0.0313 \times 2.303} = 33.61 \text{ minutes}$$

$$(t_c - t_2) = \frac{0.5080}{0.0295 \times 2.303} = 7.48 \text{ minutes.}$$

If the sample had been stored in the 75° oven for four hours the heating time (t_0 to t_1) will be found to be approximately one hundred ten minutes, thus from Eq. 19

$$\text{ETTE} = (240\text{–}110) + (33.6) + (7.5) = 171.1 \text{ minutes.}$$

After a nominal storage time of four hours, the actual

[1] The steps involved in making this substitution are the following:

(*a*) $T = (T_{eq} - (T_o - T_{eq})e^{-Z_h t})$,

by differentiation

(*b*) $dT = (T_o - T_{eq})e^{-Z_h t}(Z_h)dt$.

Equation (*a*) may be rearranged to

(*c*) $e^{-Z_h t} = \frac{T_{eq} - T}{(T_o - T_{eq})}$,

substituting Eq. (*c*) in (*b*) and simplifying, the following relationship is obtained:

(*d*) $dt = \frac{dT}{(T_{eq} - T)Z_h}$

If (*a*) and (*d*) are substituted in the heating equivalent expression (Eq. 17) the following equation results:

(*e*) $t_h = \frac{A}{Z_h k_{ov}} \int_{t_0}^{t_1} \frac{e^{-\Delta H/RT} dT}{T_{eq} - T}$

and by recalling that at times t_0 and t_1, the sample temperatures are room and oven temperature respectively, substituting these limits for t_0 and t_1 in (*e*) one obtains Eq. 22. Identical reasoning for the cooling case yields Eq. 23.

TABLE I.—SOLUTIONS OF P_h AND P_c FOR THREE TEMPERATURES ASSUMING ROOM TEMPERATURE 25°

Temperature, ° C.	P_h	P_c
45	2.308	1.241
60	2.328	0.7568
75	2.423	0.5080

effective storage time at 75° was two hours, fifty-seven minutes, an error of one hour, six minutes or 27.5% in storage time.

DISCUSSION

A great simplification has been made in the mathematical treatment given above. The diffusion of heat into a nonhomogeneous body is a much more complex function of time and temperature than that described by Eq. 1. From several hundred measurements, however, it was found that the simplified expression closely described the temperature-time relationship during the major portion of the curve. For this reason, and because it permitted a solution of the final expression to be found, the simple expression was used. Experimental evidence for this approach will be presented in the next report.

The data used in the sample calculation are not unrealistic. Exaggerated storage tests in which considerable weight is given to data obtained from samples stored less than twelve hours are being used. The values of Z and I_1 used were determined on small, single bottles of tablets and so are probably characteristic. In instances where storage tests are carried out in cartons, or in bottles containing 100 or more tablets, the error caused by slow heat conduction will be even greater.

When finding the constants for use in calculating the heating and cooling times, I_h and $(I_c - I_2)$, as shown in Eqs. 17 and 18, it is essential to determine both a rate of heating and a rate of cooling coefficient. In general these two values will be different. Cooling has been found to be slower than heating. This is due perhaps to the fact that cooling samples stand in still, room air as opposed to the forced draft heating provided by most stability ovens. The coefficients required for ETTE calculation are best determined by continuously measuring sample temperature and then graphing the logarithm of the difference between the equilibrium and sample temperature against time on semilogarithm paper. The slope of the straight portion of this graph is the figure required in Eqs. 26 and 27. This graph will also permit the determination of I_1, the time when the sample reaches oven temperature. In theory, the sample never reaches this temperature, so that for the purposes of calculation an approximation must be used. The time I_1 was approximated by the time when the reaction rate had reached 95% of its final value at equilibrium temperature. In most cases, the sample temperature at this time differs from the equilibrium by less than 1°.

From the equation and the discussion, it should be apparent that the errors introduced by slow heat conduction can be minimized in several ways. The data obtained from samples stored for long periods may be weighted more heavily than those stored for short times. This approach, while effective, is not useful for quick studies where high temperature data must be included and, because none other is available, weighted heavily. Room temperature extrapolations using the Arrhenius equation depend strongly upon high temperature data, especially in the early stages of a study. On the other hand, the fact that the heating and cooling lag is constant and independent of the duration of storage presents two other choices to the formulator: *1* as the storage time increases, the magnitude of the error caused by the conduction lag decreases, the formulator may then limit his sampling to times sufficiently long that the time error becomes negligible, or *2* he may sample and analyze his formula at any time and later correct his storage times to ETTE, using the latter for breakdown plots. These two approaches are quite separate from the more obvious one of changing the container or sample size to speed the rate of heating; however, in the situation where finished product stability is being established, container changes are undesirable.

The ETTE storage time should theoretically be more satisfactory for use in conjunction with the equations of chemical kinetics in the accurate determination of specific rate constants of drug degradation reactions at exaggerated temperatures. Due to the improved accuracy available for finding these constants, the ETTE should also permit more precise estimation of product shelf life.

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An Inverse Isotope Dilution Analysis of Salicylic Acid*

By H. A. SWARTZ and J. E. CHRISTIAN

A method is described for the inverse isotope dilution analysis of salicylic acid using iodine-131 labeled iodine monochloride as a reagent to form the iodine-131 labeled derivative, 3,5-diiodosalicylic acid.

THE determination of trace amounts of medicinal agents and various important biological substances by inverse isotope dilution methods has increased to a marked extent in recent years. The development and application of this method of assay has permitted the determination of minute amounts of materials, with a good degree of accuracy, that was impossible before its introduction. A complete historical and literature survey of the isotope dilution procedure of analysis was presented by Pinajian, Christian, and Wright (1). The procedure and limitations for each of the four types of isotope dilution analysis was described by Christian and Pinajian (2); included in this review was a report on a variation of the inverse isotope dilution method of assay, referred to as the labeled reagent addition method. This procedure is particularly suited for the determination of submilligram quantities of material and has been employed for the determination of several medicinal and biologically important compounds (3–9). The work reported here consists of a study of the analysis of salicylic acid by the labeled reagent addition method of inverse isotope dilution analysis using iodine-131 labeled iodine monochloride as the reagent. This reagent may be conveniently prepared in good yields (10) and reacts quantitatively with salicylic acid to form the 3,5-diiodosalicylic acid which may be readily isolated and purified.

EXPERIMENTAL

Iodine-131 labeled iodine monochloride was prepared and the radiochemical purity carefully determined as previously described (10). The iodine monochloride was contained in a glacial acetic acid solution the content of which was accurately determined by titration with 0.01 *N* sodium thiosulfate solution. Iodine-131 labeled 3,5-diiodosalicylic acid was prepared from the reagent by reaction with pure salicylic acid and the specific activity of the derivative carefully determined in the following manner:

One-tenth of a gram of salicylic acid was placed into each of three 5-inch test tubes. The volumes of the glacial acetic acid solution of labeled iodine monochloride that contained 0.252 Gm. of the reagent were added. The test tubes were placed into a 250-ml. beaker containing 100 ml. of water and heated gradually to 70 to 80° on a hot plate with constant stirring by means of a power stirrer. The solutions were maintained under these conditions for twenty minutes, cooled to 50°, and 3 ml. of water added with constant stirring. The precipitates that formed were heated at 40 to 50° for five minutes, and collected in a Büchner funnel with the aid of suction. The precipitates were washed with 20% acetic acid, water and, with the suction off, a 5% aqueous solution of sodium sulfite. The suction was applied after five minutes and the precipitates were dried in an oven, transferred to a 5-inch test tube, and dispersed in 1 ml. of hot water. Acetone was added drop by drop until a clear solution was obtained. The solutions were filtered and the filtrates collected in a 5-inch test tube which was set aside to allow crystallization to occur. The crystals were collected on a filter in a 2-inch funnel, washed with water and dried in an oven. The crystals were recrystallized from glacial acetic acid and again from acetone in the same manner, dried and weighed and dissolved in sufficient glacial acetic acid to produce 25 ml. One-milliliter samples of the solutions were placed into 2-inch test tubes for determining the radioactivity per unit weight. All radiometric measurements were made with a scintillation counter. The instrument employed was a Model 115 pulse height analyzer, Radiation Instrument Laboratory, Chicago, Ill. The samples contained in the 2-inch test tubes were placed in the counting well of the instrument, and held in position by passing them through an opening in a cardboard disk placed over the well, to insure geometrical reproducibility. The average specific activities of 3 samples of the 3,5-diiodosalicylic acid formed from two separate iodine-131 labeled iodine monochloride reagent solutions were found to be 6.76×10^6 and 6.48×10^6 c. p. m./Gm.

Analysis of Known Quantities of Salicylic Acid.—Using iodine-131 labeled iodine monochloride solutions of known radioactivity as determined through the iodine-131 labeled 3,5-diiodosalicylic acid as described above, six samples of known weights of salicylic acid, varying from 20 to 0.2 mg., were analyzed. This was done to test the reproducibility of the method. The salicylic acid samples were prepared by dissolving 2.0 Gm. of salicylic acid in sufficient glacial acetic acid to produce a total volume of 200 ml. Specific volumes of these solutions were taken to obtain the desired weights of salicylic acid (see Table I for quantities) and placed into 5-inch test tubes. The volume of the glacial acetic acid solution of labeled iodine monochloride that contained the desired weight of reagent for each level was added (see Table I for quantities). The test tubes were placed into a 250-ml. beaker containing 100 ml. of water and heated gradually to 70 to 80° on a hot plate. The solutions were maintained at that temperature for twenty minutes and agitated

* Received January 27, 1958, from the Bionucleonics Research Laboratory, School of Pharmacy, Purdue University, Lafayette, Ind.
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TABLE I.—THE RESULTS OF THE INVERSE ISOTOPE DILUTION ANALYSIS OF SALICYLIC ACID AT LEVELS BETWEEN 20 AND 0.2 MILLIGRAMS

Known Wt. Salicylic Acid, mg.	Weight[a] ICl, Gm.	Weight Carrier, mg.	Isolated Crystals, mg.	Sp. Act. of Diluted Derivatives, c.p.m./Gm.	Salicylic Acid Found, mg.	Recovery, %	Error, %
20	1.0	475.0	98.4	6.73×10^5	19.4	97	3
20	1.0	475.0	109.1	6.74×10^5	19.4	97	3
20	1.0	579.2	248.2	5.63×10^5	19.4	97	3
						Av. 97	Av. 3
10	0.50	475.0	113.3	3.50×10^5	9.5	95	5
10	0.50	475.0	95.5	3.51×10^5	9.6	96	4
10	0.50	406.8	160.2	4.13×10^5	9.7	97	3
						Av. 96	Av. 4
6	0.045[b]	194.7	68.3	6.00×10^5	6.7	111	11
6	0.045	475.0	65.4	2.19×10^5	5.8	96.6	3.4
6	0.045	356.1	160.9	2.81×10^5	5.7	95	5
						Av. 100.9	Av. 4.8
2	0.015[b]	194.7	43.8	2.16×10^5	2.2	110	10
2	0.015	475.0	191.9	7.43×10^4	1.9	95	5
2	0.015	316.0	141.9	1.06×10^5	1.9	95	5
						Av. 100	Av. 6.6
0.6	0.006	475.0	147.9	2.16×10^4	0.56	93.3	6.7
0.6	0.006	475.0	227.2	2.30×10^4	0.59	98.3	1.7
0.6	0.006	329.2	145.9	3.39×10^4	0.61	101.6	1.6
						Av. 97.4	Av. 3.3
0.2	0.002[b]	194.7	85.2	2.02×10^4	0.21	105	5
0.2	0.002	475.0	225.5	7.81×10^3	0.202	101	1
0.2	0.002	262.3	102.4	1.49×10^4	0.225	114	14
						Av. 106.6	Av. 6.6

[a] Weight of iodine-131 labeled iodine monochloride.

[b] In these samples the specific activity of the resultant 3,5-diiodosalicylic acid before dilution with carrier was 6.67×10^6 c. p. m./Gm. All others had a specific activity of 6.48×10^6 c. p. m./Gm.

repeatedly by hand. Carrier (unlabeled, 3,5-diiodosalicylic acid), in solution in hot glacial acetic acid, was added (see Table I for quantities). The solutions were agitated to give thorough admixture, and set aside to cool and to allow the formation of crystals. The crystals were collected on a filter in a 2-inch funnel, washed thoroughly with 20% acetic acid, and with water. The crystals were dried in an oven and recrystallized from acetone, glacial acetic acid, and again from acetone. The crystals were dried in an oven and carefully weighed on a micro balance (see Table I for weights). The crystals were dissolved in 10 to 15 ml. of hot glacial acetic acid and transferred to 10 or 25-ml. volumetric flasks, depending upon the weight of the crystals. The solutions were made up to volume with glacial acetic acid, and 1 or 2-ml. samples placed into 2-inch test tubes for the determination of the radioactivity.

The specific activities of the carrier diluted derivatives were calculated (see Table I) and in conjunction with the specific additives of the undiluted derivatives, as previously determined, were applied in the following formula for the calculation of the salicylic acid (11):

$$X = \frac{WS}{S_o - S} \times \frac{M^1}{M^2}$$

Where X equals the weight of the salicylic acid being determined, W equals the weight of carrier added, S equals the specific activity of the carrier diluted derivative, S_o equals the specific activity of the derivative before dilution with carrier, M^1 equals the molecular weight of the salicylic acid, and M^2 equals the molecular weight of the derivative, 3,5-diiodosalicylic acid. (See Table I).

SUMMARY AND CONCLUSIONS

The reaction of iodine monochloride with salicylic acid has been successfully applied to an inverse isotope dilution procedure for the analysis of salicylic acid. The method developed was applied to levels of 20 to 0.2 mg. of salicylic acid and theoretically should be sensitive to lower levels of the acid. The criteria that the compound analyzed must be quantitatively converted to the labeled derivative was met at the levels of reaction investigated.

The procedure can be applied to the analysis of acetylsalicylic acid, by first carrying out the quantitative hydrolysis of the acetylsalicylic acid to salicylic acid and continuing the procedure as described for salicylic acid.

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The Effect of Zinc Acetate on Red Corpuscles*

By DONALD E. CADWALLADER, Jr.,† and WILLIAM J. HUSA

In hemolysis experiments, unusual behavior was displayed by solutions of zinc acetate. Hemolysis of red blood cells took place at an unusually high concentration range while extremely low concentrations of zinc acetate protected red blood corpuscles against osmotic hemolysis. The results are graphically portrayed.

HARTMAN AND HUSA (1) found extremely high hemolytic *i* values for zinc sulfate. They were of the opinion that zinc sulfate strengthens the erythrocyte membrane in some way, making the red cell more resistant to hemolysis in hypotonic solution.

The purpose of this investigation was to observe the effect of a zinc salt of an organic acid on rabbit and human red blood cells.

EXPERIMENTAL

Collection of Blood, Preparation of Solutions and Quantitative Determination of Per Cent Hemolysis.—The procedures employed were essentially those described by previous workers (2, 3).

Preparation of Graphs.—Three sodium chloride experiments employing rabbit blood were carried out. The average readings of these three experiments were utilized to construct one standard sodium chloride curve, Fig. 1. The average readings of three zinc acetate experiments, using the same rabbit blood samples as the above sodium chloride experiments, were employed to construct Fig. 2. Therefore, Fig. 1 is the standard sodium chloride curve for Fig. 2, the zinc acetate curve.

Experiments were run with rabbit blood employing sodium chloride solutions and comparatively low concentrations of zinc acetate. A bell-shaped hemolysis curve was not formed with the lower concentrations of zinc acetate as was the case when higher concentrations of zinc acetate were used, Fig. 2. A dilution range of zinc acetate was employed so that approximately 5 to 100% hemolysis was obtained. Using Eq. 1, hemolytic *i* values were calculated from these zinc acetate experiments. These hemolytic *i* values are shown in Table I.

TABLE I.—VALUES FOR *i* FOR ZINC ACETATE AT CONCENTRATIONS, IN GM./100 ML., CAUSING 25, 50, AND 75% HEMOLYSIS OF RABBIT AND HUMAN ERYTHROCYTES[a]

Blood	Hemolysis, % 25	50	75	Average
Rabbit	718	835	902	818
Human	58.3	568	648	...

[a] All *i* values represent an average of two blood samples.

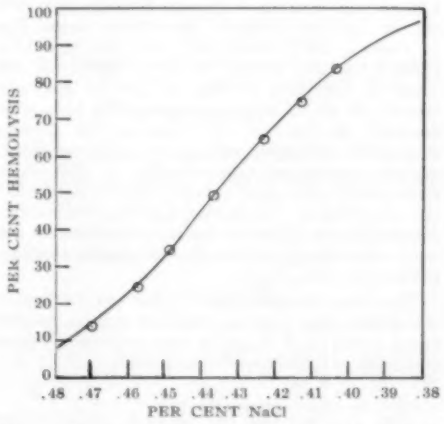

Fig. 1.—Standard sodium chloride curve for hemolysis of rabbit erythrocytes.

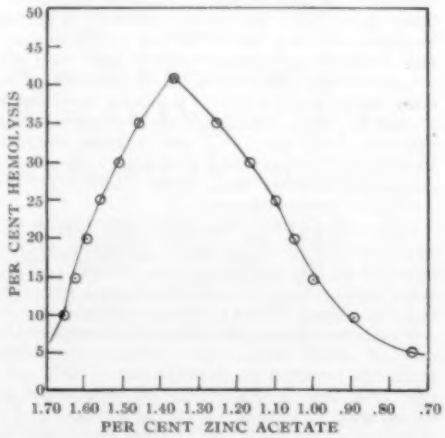

Fig. 2.—Hemolysis of rabbit erythrocytes in zinc acetate solutions of varying concentrations.

The construction of Fig. 3 consisted of two parts. The left hand portion of the curve is Fig. 2 transposed to the log scale. In order to make up the right hand portion of the curve, the Gm./100 ml. of zinc acetate causing 25, 50, and 75% hemolysis, at comparatively low zinc acetate concentrations, had to be calculated with reference to Fig. 1. Utilizing the following equation

* Received January 4, 1958, from the College of Pharmacy, University of Florida, Gainesville.

This paper is based in part upon a dissertation presented to the Graduate Council of the University of Florida by Donald E. Cadwallader, Jr., in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

† Fellow of the American Foundation for Pharmaceutical Education 1954–1957. Research Associate, Sterling-Winthrop Research Institute, Rensselaer, N. Y.

Presented to the Pharmacy Section, A. A. A. S., Indianapolis meeting, December, 1957.

$$\frac{\begin{pmatrix} i \text{ value for} \\ \text{NaCl} \end{pmatrix}\begin{pmatrix} \text{Gm. of NaCl} \\ \text{in 100 ml.} \\ \text{of solution} \end{pmatrix}}{(\text{Gm.-molecular weight of NaCl})} = \frac{\begin{pmatrix} i \text{ value for} \\ \text{zinc} \\ \text{acetate} \end{pmatrix}\begin{pmatrix} \text{Gm. of zinc} \\ \text{acetate in 100} \\ \text{ml. of solution} \end{pmatrix}}{(\text{Gm.-molecular weight of } Zn(Ac)_2)} \quad \text{(Eq. 1)}$$

the Gm./100 ml. of zinc acetate causing 25 per cent hemolysis was calculated by substituting 1.86 for the i value of sodium chloride, Gm./100 ml. of sodium chloride, determined from Fig. 1, causing 25% hemolysis, and the hemolytic i value for zinc acetate for 25% hemolysis (see Table I), and then solving for Gm. of zinc acetate in 100 ml. of solution causing 25% hemolysis. Similarly the Gm./100 ml. of zinc acetate, causing 50 and 75% hemolysis, were calculated by substituting the appropriate values in Eq. 1. The above three points were used to construct the right hand portion of Fig. 3. In this manner, Fig. 3 becomes a compilation of all the zinc acetate experiments run with rabbit blood, and Fig. 1 is the standard sodium chloride curve for Fig. 3.

The same procedure was carried out for human blood using Fig. 4 as the standard sodium chloride curve. Figures 4, 5, and 6 were constructed from the average of four experimental runs.

DISCUSSION

In the hemolysis experiments, unusual behavior was displayed by solutions of zinc acetate. Figure 2 shows the hemolysis curve when rabbit erythrocytes were placed in various concentrations of zinc acetate. Starting in 1.7% zinc acetate solution, hemolysis increased to a maximum of 42% in 1.37% zinc acetate solution and then decreased until only 5% hemolysis was observed in 0.7% solution. The same behavior was noticed for human blood and is plotted in Fig. 5. However, the maximum hemolysis was 95% in 1.10% zinc acetate solution. Hemolysis of human blood took place over a lower concentration range than rabbit blood; 1.60 to 0.40% zinc acetate solution.

Zinc acetate solutions ranging from 0.70 to 0.01% displayed essentially complete protection against hemolysis of rabbit erythrocytes. Human erythrocytes were protected in solutions ranging from 0.40 to 0.1%. Upon further lowering of the zinc acetate concentration, hemolysis increased rapidly in the case of rabbit blood. For human blood 25% hemolysis occurred in approximately 0.05% solution, then there was a leveling off with hemolysis commencing to increase rapidly at a concentration of 0.01%. Extremely high i values were found for zinc acetate using data obtained from the hemolysis experiments. Higher hemolytic i values were found for rabbit blood.

The pH of zinc acetate solutions used in hemolysis experiments was slightly acid. No unusual deviations of pH were observed for the various solutions.

In 1931 Jaeger (4) reported that the hemolysis of horse erythrocytes by saponins or water was inhibited by zinc acetate. He also stated that chicken protein and horse serum were precipitated and that

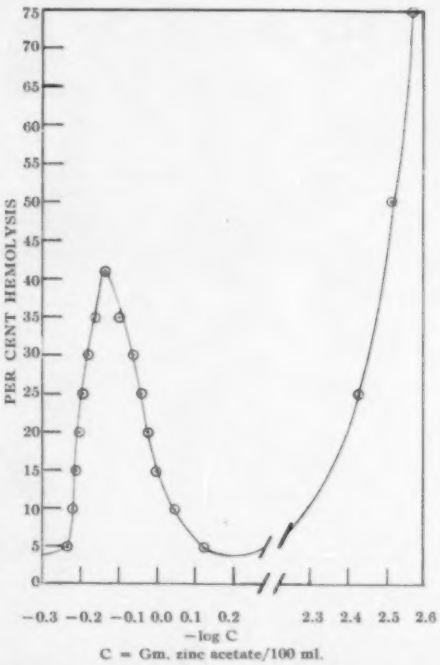

Fig. 3.—Hemolysis of rabbit erythrocytes in zinc acetate solutions of varying concentration.

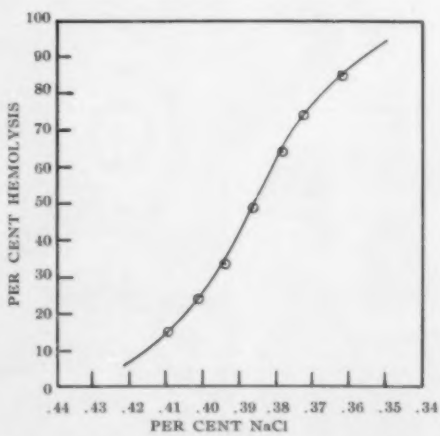

Fig. 4.—Standard sodium chloride curve for hemolysis of human erythrocytes.

lipoids (cholesterol and lecithin) were almost quantitatively precipitated by zinc ion. Hartman and Husa (1) found unusually high i values for zinc sulfate.

Red cells take up zinc very readily (5). Whether the zinc enters the red cell or is bound to the membrane is not known, but it cannot be removed by washing with isotonic saline. Gurd and Wilcox (6)

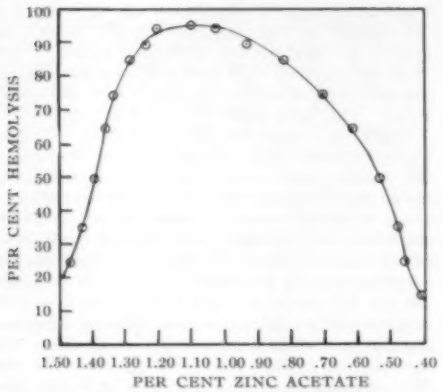

Fig. 5.—Hemolysis of human erythrocytes in zinc acetate solutions of varying concentrations.

list the postulates which lie behind the computations for the binding theory of zinc-albumin systems at 0°. The postulates are: (*a*) Zinc ion is bound to only one class of sites within the experimental range of pH. (*b*) The interdependence of zinc ion binding and pH reflects a competition between zinc ion and hydrogen ion for the same ligand groups. (*c*) The ligand groups in question are the imidazole side chains of the histidine residues. (*d*) Complex formation between zinc ion and each of the imidazole groups of the albumin molecule occurs in the ratio of 1:1.

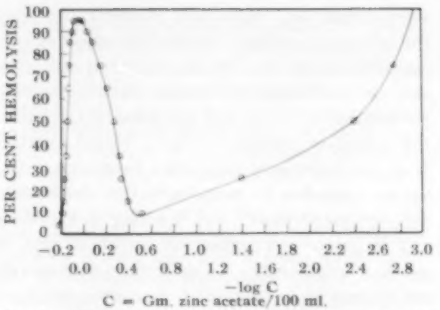

Fig. 6.—Hemolysis of human erythrocytes in zinc acetate solutions of varying concentrations.

SUMMARY

1. In hemolysis experiments with rabbit and human blood, unusual behavior was observed for zinc acetate.

2. Extremely high *i* values were calculated for zinc acetate using hemolytic data.

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Isotonic Solutions VI. The Permeability of Red Corpuscles to Various Salts of Organic Acids*

By DONALD E. CADWALLADER, Jr.,† and WILLIAM J. HUSA

Employing the hemolytic method, van't Hoff *i* values were obtained for various salts of organic acids. Results indicate that sodium and potassium salts of citric, tartaric, and succinic acids have higher *hemolytic i* values than might be expected. Ammonium citrate and tartrate have higher *i* values than the corresponding sodium and potassium salts. Ammonium acetate, benzoate, and salicylate, and bismuth potassium tartrate caused 100 per cent hemolysis in all concentrations tested. This illustrates the need for caution in the use of isotonic coefficients obtained from colligative properties alone.

THE PREPARATION of solutions isotonic with human blood is of prime importance to the safety and comfort of the patient. Much information has been compiled concerning the use of colligative properties of drugs in the calculation of isotonic solutions.

Working with isotonic solutions, Husa and Adams (1) pointed out that certain substances at concentrations calculated to be isotonic with blood, according to physicochemical data, did not prevent hemolysis. A hemolytic method for the quantitative determination of sodium chloride equivalents was employed by Husa and Grosicki (2), and isotonic coefficients or *i* values of various amino acids, sugars, and salts

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were calculated. Husa and Easterly (3), using the hemolytic method, studied the effect of urea and urea derivatives on the red blood cell. The isotonic coefficients of various electrolytes were determined by Husa and Hartman (4), applying the hemolytic method. The i values obtained from the hemolytic tests were compared with i values calculated from activity coefficients, osmotic coefficients, and freezing point data.

The purpose of this investigation was to conduct experiments to determine the isotonic coefficients of various salts of organic acids and to study their effect on red blood cells.

SIGNIFICANCE OF ACTIVITY TERMS

The van't Hoff i and the osmotic coefficient are used to cover all types of deviation from ideal behavior. The isotonic coefficient, van't Hoff i, may be defined as the ratio of the colligative effect produced by a given molality of an electrolyte to the effect observed for the same molality of a nonelectrolyte (5). The osmotic coefficient, introduced by Bjerrum (6), is defined as the ratio of an observed colligative property to what the property would be if dissociation were complete. The equation relating the osmotic coefficient (g) to the van't Hoff i is

$$g = \frac{i}{\gamma} \qquad \text{(Eq. 1)}$$

where γ is the number of ions in a formula weight.

The terms i and g as used for nonelectrolytes and electrolytes in solution indicate the degree of dissociation. In solutions of nonelectrolytes i and g both have a maximum value of one. In solutions of electrolytes the value of i is determined principally by the number of ions into which the molecule can dissociate and the charge on these ions. At infinite dilution the value of i is identical with the number of ions into which a molecule of the solute can dissociate. For example, sodium chloride can dissociate into two ions; at infinite dilution it would have an i value of two. Under the same conditions, the value of g for sodium chloride would be one, the i value divided by the number of ions per formula weight.

In the case of electrolytes in solution, the values of i and g depend upon the electrolytic dissociation or ionic activity of the electrolyte. The terms i and g as used throughout this investigation indicate the degree of dissociation. The terms hemolytic i and hemolytic g values carry the added significance of being a measure of hemolytic activity. That is to say, a higher i value for a substance would indicate that this substance affords greater protection against hemolysis of a red blood cell than a substance, with the same number of ions per formula weight, with a lower i value. Conversely, a lower i value for a substance would indicate greater hemolytic activity than a substance, having an equal number of ions, with a higher i value. The hemolytic activity might be defined as the ability of a substance to release hemoglobin from an erythrocyte. For example, let us, for some compound, prepare a series of solutions of varying concentration. For some other compound a series of solutions of varying concentration, equimolar with respect to the first series, is set up. If an equal portion of blood is added to each of these solutions, hemolysis will be greater in the solutions containing the compound with the lower i value. The same would be true of values of g.

Higher or lower hemolytic i values (i values higher or lower than that predicted on the basis of the limiting law) would have to be attributed to either the selective permeability of the red cell membrane to the anions and/or cations in solution, or to the alteration of integrity of the cell by some action of the substance on the erythrocyte membrane. A substance having ions that do not easily permeate the red cell membrane would be expected to have comparatively higher i values than a substance having ions which permeate the membrane. Extremely high i values were found for zinc sulfate by Hartman and Husa (4). This may be due to some action of the zinc on the cell membrane to make it more resistant to hemolysis.

The Debye-Huckel limiting law states that the departure from ideal behavior in a given solvent is governed by the ionic strength of the medium and the valences of the ions of the electrolyte, but is independent of their chemical nature (7). The valence type of the electrolyte should thus be the essential factor in determining its activity coefficient at a given ionic strength. Therefore a 1:1 electrolyte, like sodium chloride, could have a maximum value of two for van't Hoff i and a maximum value of one for g. However, in many cases the i values, determined by the hemolytic method, are higher than the limiting law predicts. Thus it would seem practical to propose and advocate the use of the terms *hemolytic* i value and *hemolytic* osmotic coefficient (g) for these values that are calculated from biological data, i.e., hemolytic method.

It should be kept in mind that all the hemolytic i values calculated in this investigation have been determined using sodium chloride as the standard. In other words, all the hemolytic i values are based on calculations using 1.86 as the van't Hoff i for sodium chloride.

EXPERIMENTAL

Collection of Blood.—The blood used in the present investigation was obtained in the same manner as described previously by Husa and co-workers (2, 3). The human blood used was obtained mainly from the veins of the arms of a twenty-five year old male. Several experiments were carried out employing blood from helpful volunteers.

Preparation of Solutions.—Depending on solubility, 10 or 5% stock solutions of the salts were prepared so that the solutions contained 10 or 5 Gm. of the anhydrous salt/100 ml. of solution. Dilutions were made in the same manner as described by other workers (2, 3).

Quantitative Determination of Per Cent Hemolysis.—The calculation of hemolytic i values has been described in detail by Grosicki and Husa and Easterly and Husa (2, 3).

DISCUSSION

Variability of Blood.—As in the case of previous investigations (2–4), the hemolysis occurring in

various concentrations of sodium chloride was determined for each sample of blood that was used in carrying out hemolytic experiments. Thus any error due to variable erythrocyte fragility was avoided, since any differences in red corpuscle fragility were taken care of by comparison of hemolysis in a given solution with hemolysis in sodium chloride.

Features used to decrease experimental error were the use of duplicate tubes of all solutions in each experiment, and employment of five separate tubes for each blood sample for 100% hemolysis, three at the beginning and two at the end of the experimental run.

At least two experiments, using two different blood samples of the same species, were carried out for each salt.

Results with Citrate, Tartrate, and Succinate Salts.—Slightly higher hemolytic i values than would be expected, i values greater than four for the citrate salts and greater than three for the tartrate and succinate salts, were obtained for the sodium and potassium salts of citric, tartaric, and succinic acids. The sodium salts of succinic, tartaric, fumaric, and citric acids are known to be fairly indifferent to various cells and tissues (8). This behavior can be ascribed to several reasons. These acids are fairly strong organic acids, and as such, are lipoid insoluble. In solution, the ions are strongly hydrated due to the polar carboxylic and hydroxyl groups. Therefore, they diffuse slowly and traverse pores with difficulty. In addition they compete with colloids for water, they are surface inactive, and lack "hydrotropic" properties.

The higher i values of the citrate, tartrate, and succinate salts may be explained by the fact that these polyvalent anions are distinctly less able to permeate the cell membrane than other anions, i.e., monovalent (8). Although the red cell is generally thought of as being impermeable to sodium ion, this ion is able to diffuse slowly and in small proportions to the interior of the cell (9, 10). If a sodium ion can traverse the membrane, then a chloride ion could follow, or a potassium ion could come out of the cell in order to maintain electrical neutrality. If a sodium and chloride ion entered the cell, then there would be an influx of water. However, in the case of sodium citrate, the anion cannot diffuse through the membrane, so that there can be no immediate change in concentration. This phenomenon could account for sodium citrate and other similar acting salts having higher hemolytic i values than might be expected. Basing the calculations on standard sodium chloride solutions having a value of 1.86 for the van't Hoff i, trisodium citrate might be expected to have an i value close to 3.72 (2×1.86), if dissociation for citrate salts was as complete as sodium chloride. Actually, this calculated value of 3.72 is high since the third dissociation of citrate salts is relatively weak. The hemolytic i values of the citrate salts studied were greater than four. In addition, it is known that isotonic solutions of inorganic salts, with the exception of ammonium salts, are osmotically indifferent to red corpuscles. Hober (8) states that this is true only when monovalent anions are concerned in the exchange, for in isosmotic solution of polyvalent anions, blood corpuscles shrink. He further explains that in order to maintain electroneutrality a divalent anion, for instance, exacts two chloride or two bicarbonate ions in exchange. When this takes place the osmotic resistance of the cell rises. This is probably due mainly to the dehydrating effect of the polyvalent anions which results in a closer packing of the structural components of the surface membrane. It has been shown that an increase in dehydration will be indicated by a decrease of the spacing in protein and lipoprotein molecules (11). This dehydrating effect, coupled with the greater hydration of these ions, could account for the resistance of the cell membrane to hemolysis or, to say it another way, account for the seemingly greater protective effect against hemolysis of the red cells by the organic salts as compared to most inorganic salts. Any one or a combination of the above factors could account for high i values calculated from hemolytic data.

Addition of 0.2% sodium chloride to potassium tartrate solutions did not lower the hemolytic i value significantly. The i value in this case still remained higher than three.

The curves obtained by plotting the per cent hemolysis of most of the citrate, tartrate, and succinate salts against per cent concentration were not sigmoid in character, as was the case with inorganic salts (4, 12). The slopes of the curves were not as great as that of inorganic salts since 25, 50, and 75% hemolysis of blood cells took place over a much wider range of concentration than is normally found with inorganic salts. The smaller slope for these curves of organic salts results in increasingly higher hemolytic i values as calculated from concentrations causing 25, 50, and 75% hemolysis, respectively. Goyan, *et al.* (13), have graphically shown the average values of the molal lowering of freezing point for various types of substances. The molal freezing point depression is plotted against increasing molal concentration of the solute. The graph depicts that at more dilute solutions, especially lower than 0.1 molal, the freezing point depression increases. This is particularly true of uni-divalent electrolytes and uni-trivalent electrolytes. There is an increasing negative slope of these curves as the concentration decreases from 0.1 molal to infinite dilution. This increase of molal freezing point depression, which is probably due to increased activity of the ions in a more dilute solution, could correspond to the increase in the hemolytic i values over a dilution range going from a higher dilution causing 25% hemolysis to a lower concentration causing 75% hemolysis of erythrocytes in solutions of di- and tricarboxylic acid salts. (See Tables I and II.)

The ammonium salts of citric and tartaric acids displayed much higher i values than the corresponding sodium and potassium salts. Ammonium citrate being a dibasic salt of a weak base and a fairly strong organic acid is, as would be expected, acid in solution. Measurements of pH show that solutions ranging from 1.0 to 0.25% have a pH of 5.10 to 5.32. There was very little change after the addition of rabbit blood. Ammonium tartrate solutions have a pH around 6.50. The addition of blood causes an increase in pH, but this pH is still slightly acid. The higher hemolytic i values can be attributed to the fact that the degree of hemolysis was most likely retarded by the acidic nature of the ammonium

TABLE I.—VALUES OF *i*, FOR VARIOUS ORGANIC SALTS, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF RABBIT ERYTHROCYTES[a]

Salt	Hemolysis, % 25	50	75	Averages
Ammonium citrate (dibasic)	3.42	4.45	6.31	4.73
Ammonium tartrate	3.09	3.13	3.27	3.16
Antimony potassium tartrate	1.38	1.43	1.50	1.44
Calcium acetate	3.05	3.11	3.22	3.12
Calcium gluconate	2.45	2.61	2.67	2.58
Calcium levulinate	3.09	3.17	3.24	3.17
Potassium acetate (4)	1.49	1.51	1.54	1.51
Potassium citrate	3.95	4.02	4.15	4.04
Potassium sodium tartrate[b]	3.14	3.21	3.31	3.22
Potassium tartrate	3.25	3.26	3.28	3.26
Sodium acetate (2)	1.79	1.78	1.77	1.78
Sodium benzoate	1.76	1.75	1.75	1.75
Sodium citrate	4.06	4.17	4.30	4.18
Sodium *para*-aminobenzoate	1.98	1.96	1.92	1.95
Sodium salicylate	1.78	1.80	1.78	1.79
Sodium succinate	3.27	3.34	3.36	3.32
Sodium tartrate[b]	3.27	3.34	3.49	3.36

[a] Unless otherwise indicated all *i* values represent an average of two blood samples.
[b] Average of three blood samples.

TABLE II.—VALUES OF *i*, FOR VARIOUS ORGANIC SALTS, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF HUMAN ERYTHROCYTES[a]

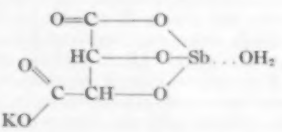
Salt	Hemolysis, % 25	50	75	Averages
Ammonium citrate (dibasic)	3.39	4.54	6.83	4.92
Ammonium tartrate	3.37	3.55	3.74	3.55
Antimony potassium tartrate	1.49	1.59	1.67	1.58
Calcium acetate	2.60	2.81	3.01	2.80
Calcium gluconate	2.66	2.79	2.96	2.80
Calcium levulinate	3.09	3.30	3.52	3.30
Potassium acetate (4)	1.58	1.57	1.57	1.57
Potassium citrate	4.03	4.18	4.34	4.18
Potassium sodium tartrate	3.18	3.28	3.44	3.30
Potassium tartrate	3.22	3.38	3.49	3.36
Potassium tartrate[b]	3.15	3.20	3.33	3.23
Sodium benzoate	1.85	1.85	1.85	1.85
Sodium citrate	3.90	4.05	4.12	4.02
Sodium *para*-aminobenzoate	1.94	1.93	1.96	1.94
Sodium *para*-aminosalicylate	2.00	2.00	1.99	2.00
Sodium salicylate	1.88	1.89	1.87	1.88
Sodium succinate	3.13	3.25	3.43	3.27
Sodium tartrate[c]	3.24	3.34	3.54	3.37

[a] Unless otherwise indicated all *i* values represent an average of two blood samples.
[b] In presence of 0.2% sodium chloride.
[c] Average of three blood samples.

solutions. This is supported by Gibson (14) who points out that in survival studies of red blood cells for transfusion, the degree of swelling, spontaneous hemolysis, and degree of osmotic fragility was retarded best by acid citrate-dextrose solution (pH 5.5) as compared to sodium citrate solution and sodium citrate-dextrose solution (both pH 7.1–7.4.).

Erratic results were obtained with ammonium succinate solutions. Although the sodium chloride hemolysis was almost identical for three different blood samples, the hemolysis of cells in the same concentrations of ammonium succinate solutions increased with each successive run. The degree of hemolysis seemed to increase with the age of the solutions. There was no apparent change in the solutions as far as pH was concerned. No further evaluation was carried out with ammonium succinate.

Antimony potassium tartrate has a hemolytic *i* value of 1.44 for rabbit blood and 1.58 for human blood. It appears to be acting as a weakly dissociated salt having two ions in a formula weight. Several authors (15, 16) have found evidence of a cyclic structure that would give rise to only two ions in solution. The formula is as follows:



High concentrations of antimony in red cells following administration of antimony potassium tartrate and other antimonial drugs has been noted (17, 18). Using radioactive techniques, Bahner (19) has shown that antimony can diffuse through the red cell membrane and attach itself to the globin portion of hemoglobin.

Bismuth potassium tartrate appears to penetrate the rabbit and human erythrocyte in all concentrations tested. The addition of 0.6% sodium chloride to the solutions prevented the hemolysis of erythrocytes by the bismuth salt.

Results with Sodium Salts of Various Organic Acids.—Osmotic coefficients were calculated for the various sodium salts from their hemolytic *i* values. The hemolytic osmotic coefficient served the purpose of equating all the salts, whether mono-, di-, or trivalent, to a common factor of one. Graphs were prepared in which the per cent hemolysis in increasing order was plotted along the abscissa, and the osmotic coefficient (*g*) was plotted in increasing order along the ordinate. Since all of the compounds were sodium salts, the cation was held constant.

Figures 1 and 2 depict the effect of various organic anions on rabbit and human blood. The order in which anions appear to protect rabbit erythrocytes against hemolysis is as follows: tartrate > succinate > citrate > *p*-aminobenzoate > acetate > salicylate > benzoate. Since some of the values for various organic anions fall fairly close together, it might be better to separate these anions into several groups. The order in which the anions protect against hemolysis of rabbit erythrocytes would then be; tartrate, succinate > citrate > *p*-aminobenzoate > acetate, salicylate, benzoate.

For human blood the order of protection against hemolysis is tartrate > succinate > *p*-aminosalicylate > *p*-aminobenzoate > salicylate > benzoate. Citrate falls between succinate and *p*-aminobenzoate depending on the concentration. (See Table III.)

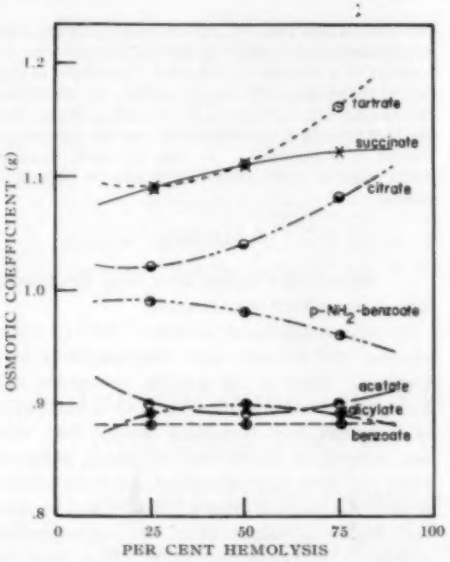


Fig. 1.—Comparison of hemolytic activity of sodium salts of various organic acids (rabbit erythrocytes).

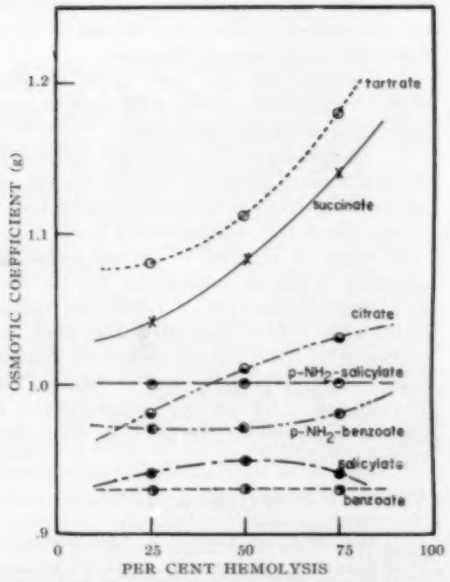


Fig. 2.—Comparison of hemolytic activity of sodium salts of various organic acids (human erythrocytes).

The aromatic acid salts acted very much like inorganic salts in that their individual curves (per cent hemolysis against per cent concentration) were sigmoid, and 25, 50, and 75% hemolysis took place over a relatively small concentration range. In addition, the hemolytic i values, calculated for 25, 50, and 75% hemolysis, did not vary more than 1 or 2% from each other.

TABLE III.—VALUES FOR OSMOTIC COEFFICIENTS (g), CALCULATED FROM HEMOLYTIC i VALUES, OF SODIUM SALTS OF VARIOUS ORGANIC ACIDS

Salt	Blood	Hemolysis, % 25	50	75
Sodium acetate	Rabbit	0.90	0.89	0.89
Sodium benzoate	Rabbit	0.88	0.88	0.88
Sodium citrate	Rabbit	1.02	1.04	1.08
Sodium *p*-amino-benzoate	Rabbit	0.99	0.98	0.96
Sodium salicylate	Rabbit	0.89	0.90	0.89
Sodium succinate	Rabbit	1.09	1.11	1.12
Sodium tartrate	Rabbit	1.09	1.11	1.16
Sodium benzoate	Human	0.93	0.93	0.93
Sodium citrate	Human	0.98	1.01	1.03
Sodium *p*-amino-benzoate	Human	0.97	0.97	0.98
Sodium *p*-amino-salicylate	Human	1.00	1.00	1.00
Sodium salicylate	Human	0.94	0.95	0.94
Sodium succinate	Human	1.04	1.08	1.14
Sodium tartrate	Human	1.08	1.11	1.18

In general the protective effect of the organic anions seemed to increase with an increase of polar groups on the anion. This might be due to one or a combination of several possible factors. The more polar groups on an anion may lead to greater hydration of this ion. The increasing hydration could result in slower diffusion and more difficult traversing of pores (8). Hydration of the anion might decrease the actual amount of water in a given solution and thereby increase the effective concentration of the solute, thus raising the tonicity of the solution. The fact that the polyvalent anions have a dehydrating effect could be an important factor (8). This effect was discussed earlier in this paper. Hober (20) states that, as a rule, the hydrotropic character of the organic ions is diminished by increasing the number of hydroxyl or carboxyl groups, because these hydrophilic groups antagonize the organophilic effect of the molecule, i.e., tartrate is less hydrotropic than succinate. Therefore, the less hydrotropic, the less hemolytic activity. As in the case of nonpenetrating nonelectrolytes (8), the erythrocyte may suffer a leakage of electrolytes in solutions of polyvalent organic salts. This exosmosis would cause a decrease in concentration of electrolyte within the cell and an increase of electrolyte in the external solution. The possibility of certain organic anions altering the cell membrane in some way, either by some physical or chemical action or combination, should not be ruled out.

Results with Ammonium Salts.—The effect of ammonium citrate, tartrate, and succinate on the hemolysis of blood is discussed in a previous section. Isosmotic solutions of ammonium citrate and tartrate do not hemolyze blood corpuscles. Although the results with ammonium succinate were erratic, this salt did show some signs of preventing hemolysis, especially when the solutions were used shortly after preparation.

Ammonium acetate, benzoate, and salicylate caused 100% hemolysis of rabbit and human erythrocytes in concentrations of the salts ranging from 0.5 to 10%. Hemolysis took place in concentrations much higher than would be predicted from osmotic

calculations. The pH of ammonium benzoate was slightly acid with little change after the addition of rabbit blood.

Jacobs and Stewart (21) state that the ability of the ammonium salts of various acids to enter living cells usually parallels rather closely that of the free acids from which they are derived. Thus, the acetate, bicarbonate, benzoate, and other similar salts of weak acids penetrate all cells with great ease. The above authors give evidence that the mechanism of penetration of ammonium salts of weak acids is by means of the products of hydrolysis of the salt, ammonia and free acid.

The hemolytic action of ammonium salicylate is comparable to that of ammonium chloride (8). In contrast to salts of weak acids, ammonium salicylate is a salt of a strong organic acid (pK equals 3) and as such is not subject to much hydrolysis. However, the free salicylic acid is highly lipoid soluble which might account for its ease of penetration (8). Furthermore Jacobs and Stewart (21) have shown that ammonium salts of strong acids will cause hemolysis. They account for this by the fact that red blood cells are normally permeable to anions in contrast to other cells. Therefore, in the case of, say, ammonium chloride, ammonia will penetrate as NH_3 and chloride as the ion, so that there is no impermeable substance present to prevent the erythrocytes swelling and bursting.

The protection of red corpuscles against hemolysis by ammonium citrate and tartrate, and somewhat by ammonium succinate, is probably due to the impermeability of the erythrocyte membrane to the anions of these salts. It is only necessary for a membrane to be impermeable to a single species of ion, i.e., anion or cation, for it to be impermeable to the salt of which this ion is a part (22).

Results with Calcium Salts.—Since hemolysis usually took place over a wide concentration range, the individual hemolysis curves (concentration *versus* hemolysis) for calcium acetate, gluconate, and levulinate were not sigmoid. The hemolytic i values for each calcium salt, calculated from data causing 25, 50, and 75% hemolysis of human blood, increased by 0.2 going from 25 to 50% hemolysis, and another 0.2 going from 50 to 75% hemolysis. Hemolytic i values calculated from rabbit blood data did not differ to such an extent. Calcium levulinate displayed high i values for rabbit and human blood. Slightly high i values were found for calcium acetate using rabbit blood.

The high values of i for the levulinate salt may be attributed to one or more of the many explanations previously given for some of the organic anions: hydration, dehydrating effect, exosmosis, etc. However, the behavior of some of the calcium salts should probably be imputed to the calcium ion. Calcium does not cross the red cell membrane under normal conditions (23). Davson and Danielli (24) have shown that calcium ion in concentrations greater than 0.01 M accelerates the penetration of potassium into the cell and increases loss of sodium from the cat erythrocyte. Cat erythrocytes have sodium as the intracellular cation and potassium as the extracellular cation, opposite to the human red blood cell (25). Davson and Danielli also point out that unusual responses of cell membranes take place in the presence of alkaline earth ions. Ruyssen and Croes (26) found that alkaline earths appear to thicken the wall of the red cell by forming insoluble salts with phosphatide acids. Hober (27) claims that the concept of a solidifying influence of calcium is suggested by innumerable observations. In discussing calcium-protein complexes, Klotz (28) explains that the best binding is determined by specific groups and unique configurations. As can be seen, calcium could play a very complicated role in hemolysis studies.

SUMMARY

1. Hemolytic i values have been determined for various salts of organic acids.

2. The sodium and potassium salts of citric, tartaric, and succinic acids had unusually high i values. Most of the sodium, potassium, and ammonium salts of the di- and tricarboxylic acids tested had hemolysis curves that were not sigmoid, as in the case with most inorganic salts, but were more elongated, having a smaller slope. Ammonium citrate (dibasic) and tartrate had higher i values than the corresponding sodium and potassium salts. This may be attributed to the acidity of their solutions.

3. The hemolytic activity of sodium salts of various organic acids is graphically correlated. The protection of corpuscles against hemolysis by the various sodium salts seemed to be a function of polar groups on the anions.

4. Calcium salts gave fairly normal results. High i values were calculated for calcium levulinate using rabbit and human blood.

5. Ammonium acetate, benzoate, and salicylate and bismuth potassium tartrate caused 100 per cent hemolysis in all concentrations tested. It was found that concentrations much higher than would be expected to prevent hemolysis, according to osmotic calculations, had no effect in preventing hemolysis of rabbit and human erythrocytes. This illustrates the need for caution in the use of isotonic coefficients obtained from colligative property measurements alone. The addition of 0.6 per cent sodium chloride to bismuth potassium tartrate solutions did protect against hemolysis.

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Isotonic Solutions VII. The Permeability of Red Corpuscles to Various Alkaloidal Salts*

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The hemolytic method has been employed to determine the isotonic coefficient, *i*, for various alkaloidal salts. It was thought that these data would be of value in the preparation of solutions isotonic with blood, since methods based on physicochemical data are not always reliable. Direct determinations were made of the degree of hemolysis occurring in solutions of various alkaloidal salts as compared with the degree of hemolysis in solutions of sodium chloride. Experiments were conducted using rabbit and human erythrocytes.

ALTHOUGH the hemolytic method has been used extensively in permeability studies, little application of the method has been made in the study and preparation of isotonic solutions. Wokes (1) determined the ratio isotonic concentration/hemolytic concentration for a number of inorganic substances, and stated that knowing this ratio it is possible to estimate the isotonic concentration of an unknown solution by diluting the latter until it just produces hemolysis. He concluded that if a solution diluted with half its volume of distilled water produces hemolysis it is not isotonic. Husa and Adams (2), employing a more rigid technique than that used by Wokes, showed that hemolysis of red blood cells is not prevented by certain substances at concentrations calculated to be isosmotic with blood according to methods based on colligative properties. Grosicki and Husa (3) determined *i* values for various amino acids and sugars quantitatively by using the hemolytic method. This method was also employed by Easterly and Husa (4) in a quantitative study of the degree of hemolysis of rabbit and human erythrocytes in solutions of urea and urea derivatives. Hartman and Husa (5) compared the *i* values of various electrolytes determined from hemolytic tests with those calculated from freezing point data and activity coefficients. The isotonic coefficients for various salts of some organic acids have been determined by Cadwallader (6) from data obtained by the hemolytic method.

In the present investigation the *i* values of various alkaloidal salts were determined quantitatively by use of the hemolytic method. Experiments were also conducted to determine the effect that the alkaloidal salts have in preventing hemolysis when used in the presence of 0.2% sodium chloride. Hober (7) pointed out that in solutions of "indifferent" nonelectrolytes, erythrocytes undergo severe alterations which, however, could be reduced by adding small amounts of an electrolyte, such as sodium chloride. Other determinations were made in the presence of 0.6% sodium chloride to find whether any of the salts increased erythrocyte fragility even when the salt concentration was sufficient to prevent such hemolysis if no other substance were present.

EXPERIMENTAL

Collection of Blood.—The blood used in the various experiments was obtained in exactly the same

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manner as previously described by Easterly and Husa (4). The human blood samples were obtained from veins of the arm of a 24-year-old male, a 20-year-old female, and a 32-year-old male.

Preparation of Solutions.—The solutions used in this investigation were prepared in the same manner as described by Easterly and Husa (4).

Quantitative Determination of Per Cent Hemolysis.—A detailed description of the hemolytic method employed throughout the present investigation has been previously reported by Grosicki and Husa (3).

Calculation of *i* Values.—Knowing the concentrations of sodium chloride and any other compound causing the same degree of hemolysis, the value of *i* (isotonic coefficient) for the other compound was calculated according to the osmotic equation used by Grosicki and Husa (3).

In the experiments conducted in the presence of 0.2% sodium chloride, the concentration of sodium chloride used in the osmotic equation was obtained by subtracting 0.2 Gm./100 cc. sodium chloride from values obtained from curves drawn for each blood sample.

DISCUSSION

Variability of Blood.—As in earlier investigations (3–6) sodium chloride solutions were employed as a standard of reference to eliminate any error due to variable erythrocyte fragility. Any difficulty of reproducibility of the biological factors involved was taken care of by making a direct comparison of the fragility in a given solution of the substance under investigation as compared with the fragility in sodium chloride solutions. Duplicate tubes of all concentrations of the substance under investigation and sodium chloride were used in each experiment. At least two experiments, using two different blood samples of the same species, were conducted for each salt tested.

Results with Caffeine.—According to osmotic calculations, a 2.0% solution of caffeine would be osmotically equivalent to a 0.27% sodium chloride solution. Since it was not possible to prepare solutions of greater than 2% caffeine concentration due to the low solubility of the compound, experiments were also conducted to determine the effect that caffeine had in preventing hemolysis when used in the presence of sodium chloride. Quantitative determinations indicated that approximately 100% hemolysis of rabbit and human erythrocytes occurred in plain caffeine solutions and in caffeine solutions containing 0.2% sodium chloride. Tables I and II indicate that caffeine may have exhibited a slight effect in preventing hemolysis of rabbit and human erythrocytes in the presence of 0.4% sodium chloride, and that the effect diminished with increasing concentrations of caffeine. No concentration of caffeine tested caused any appreciable hemolysis of either rabbit or human erythrocytes when tested in the presence of 0.6% sodium chloride.

The mechanism by which hemolysis occurs in caffeine solutions might be ascribed to the penetration of the membrane by the free base. Undissociated molecules of some weak bases readily penetrate the red cell membrane (8). Caffeine is generally considered to be a very weak base since it does not form stable salts with the strong acids. The K_b for caffeine is 4.1×10^{-14} as compared to the weak base, ammonia, which has a K_b of 1.8×10^{-5}. It is apparent from the extremely small ionization constant for caffeine that it would be present in solution almost entirely as undissociated molecules. Consequently, it seems reasonable to ascribe the hemolysis which occurs in the presence of caffeine to the penetration of caffeine into the red corpuscles.

TABLE I.—PER CENT HEMOLYSIS AT 25° OF RABBIT ERYTHROCYTES IN SOLUTIONS OF CAFFEINE OF VARYING CONCENTRATION

Caffeine, %	Plain	In 0.2% NaCl	In 0.4% NaCl	In 0.6% NaCl
0.00	...	...	84.5	0.9
0.50	99.5	98.8	72.2	1.4
0.75	100.8	95.5	73.6	1.9
1.00	101.5	98.5	74.8	2.5
1.25	...	100.7	74.7	1.4
1.50	102.0	98.7	75.8	1.8
2.00	101.0	97.2	79.5	1.5

TABLE II.—PER CENT HEMOLYSIS AT 25° OF HUMAN ERYTHROCYTES IN SOLUTIONS OF CAFFEINE OF VARYING CONCENTRATION

Caffeine, %	Plain	In 0.2% NaCl	In 0.4% NaCl	In 0.6% NaCl
0.00	...	...	12 3	0.2
0.50	101.3	101.4	5.7	0.2
0.75	104.2	102.8	9.0	0.4
1.00	101.7	103.4	8.3	0.2
1.25	102.1	101.7	8.7	0.5
1.50	102.8	100.0	11.7	0.3
2.00	103.8	99.5	11.7	0.0

Results with Alkaloidal Salts.—In Table III it can be seen that ephedrine sulfate had an *i* value of 3.53 for rabbit blood. Ephedrine hydrochloride, even in concentrations as high as 6%, failed to show any appreciable prevention of hemolysis of rabbit erythrocytes. An *i* value of 4.06 was obtained for ephedrine sulfate using human blood (Table IV). It will also be noted in the same table that ephedrine hydrochloride had a value of 0.58 for human blood. Atropine sulfate gave *i* values 3.16 for rabbit blood and 4.10 for human blood. The *i* values found for homatropine methylbromide were 2.05 using rabbit erythrocytes and 1.87 using human erythrocytes. The structurally related compound, homatropine hydrobromide, failed to show any effect in preventing hemolysis in concentrations as high as 6%.

Jacobs (9) has comprehensively discussed the permeability of erythrocytes to weak electrolytes, and termed the type of penetration as *molecular-ionic*, i. e., a molecular penetration of the free base followed by ionic exchange. The fact that the sulfate salts prevented hemolysis whereas the hydrochloride and hydrobromide salts failed to show any appreciable prevention of hemolysis suggests that the different effect of the alkaloidal salts might be ascribed to the slower penetration of the sulfate anion. Parpart (10) has stated that the univalent anions exchange across the erythrocyte membrane with great rapidity while polyvalent anions do so at a much slower rate. Davson (11) also stated that sulfate, tartrate, and citrate anions penetrate the erythrocyte very slowly in comparison with univalent anions. Parpart (10) pointed out also that

TABLE III.—i VALUES OF COMPOUNDS CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF RABBIT BLOOD

Compound	Hemolysis, % 25	50	75	Averages
Atropine sulfate	2.57	2.96	3.96	3.16
Ephedrine sulfate	3.18	3.50	3.90	3.53
Homatropine methylbromide	2.01	2.05	2.10	2.05
	(In presence of 0.2% NaCl)			
Atropine sulfate	2.27	2.22	2.25	2.25
Ephedrine sulfate	2.56	2.51	2.46	2.51
Homatropine methylbromide	1.54	1.60	1.69	1.61

TABLE IV.—i VALUES OF COMPOUNDS CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF HUMAN BLOOD

Compound	Hemolysis, % 25	50	75	Averages
Atropine sulfate	3.62	4.01	4.67	4.10
Ephedrine hydrochloride	0.56	0.58	0.60	0.58
Ephedrine sulfate	3.61	4.05	4.51	4.06
Homatropine methylbromide	1.86	1.87	1.88	1.87
	(In presence of 0.2% NaCl)			
Atropine sulfate	2.05	2.15	2.22	2.14
Ephedrine hydrochloride	0.29	0.29	0.29	0.29
Ephedrine sulfate	1.93	1.99	2.09	2.01
Homatropine hydrobromide[a]	0.15	0.13	0.12	0.13
Homatropine methylbromide	1.31	1.38	1.43	1.38

[a] These values were determined in the presence of 0.3% sodium chloride.

at pH 5 the chloride-sulfate exchange occurred one hundred times slower than at pH 8. Ephedrine sulfate and atropine sulfate gave abnormally high i values by the hemolytic method, possibly due to the sulfate ion, since Hober (12) states that in an isosmotic solution of polyvalent anions blood corpuscles shrink and at the same time the osmotic resistance rises. This increase in osmotic resistance is due to several circumstances, among others to the dehydrating effect of the polyvalent anions which results in a closer packing of the structural components of the surface membrane. This effect, in conjunction with the greater hydration of these ions and the loss of electrolytes from within the cells (3), might account for the high values of i obtained with ephedrine sulfate and atropine sulfate.

The diverse effect of homatropine hydrobromide and homatropine methylbromide may be ascribed to the difference in chemical structure of the two compounds. The tertiary amine salt, homatropine hydrobromide, apparently undergoes hydrolysis and penetrates the red cell membrane as the free base and bromide ion. The quaternary ammonium salt, homatropine methylbromide, dissociates completely in solution and behaves similarly to salts of strong acids and strong bases. Experimental results obtained by the hemolytic method indicated that homatropine methylbromide dissociated completely (Table IV).

Effect of the Addition of Electrolytes.—In Table IV it can be observed that the i value of atropine sulfate was lowered from 4.10, in experiments with atropine sulfate alone using human blood, to 2.14 when the solutions of atropine sulfate contained 0.2% sodium chloride. The i values found for ephedrine hydrochloride, ephedrine sulfate, and homatropine methylbromide were also reduced when the apparent i values of solutions of these alkaloidal salts containing 0.2% sodium chloride were determined (Table IV). The results for rabbit blood with atropine sulfate, ephedrine sulfate, and homatropine methylbromide, in the presence of 0.2% sodium chloride, were similar to the results obtained for human blood. Thus, the phenomena observed in this investigation are in agreement with that observed by previous workers (3, 5, 7), i. e., that the addition of small proportions of sodium chloride will alter abnormal effects of substances on the red blood cell.

Effect in Solutions Containing 0.6% Sodium Chloride.—Experiments were conducted to determine whether any of the alkaloidal salts increased erythrocyte fragility to the extent of allowing hemolysis even when the salt concentration was sufficient to prevent such hemolysis if no other substance were present. From Table V it can be seen that ephedrine hydrochloride, ephedrine sulfate, and atropine sulfate caused an appreciable increase in hemolysis of rabbit erythrocytes when tested with 0.6% sodium chloride. Table VI reveals that the ephedrine salts did not produce this effect with human blood, whereas atropine sulfate and homatropine hydrobromide did cause hemolysis when used in the presence of 0.6% sodium chloride.

Difference in Results with Rabbit Blood and Human Blood.—Higher i values were obtained for atropine sulfate and ephedrine sulfate with human blood than with rabbit blood (Tables III and IV). It will also be noted in the same tables that the values reported for homatropine methylbromide have the reverse order of the i values listed for the other two alkaloidal salts. Examination of Tables III and IV also reveals that the lowering of the i values by the addition of a small proportion of an electrolyte is not as pronounced with rabbit blood as with human blood. The application of perme-

TABLE V.—HEMOLYSIS AT 25° OF RABBIT ERYTHROCYTES IN SOLUTIONS CONTAINING 0.6% SODIUM CHLORIDE AND VARIOUS PROPORTIONS OF AN ALKALOIDAL SALT

Alkaloidal Salt, %	NaCl, %	Ephedrine HCl	Ephedrine Sulfate	Atropine Sulfate	Homatropine Methylbromide
0.00	0.60	0.5	1.2	0.9	0.0
0.40	0.60	...	5.0	...	...
0.50	0.60	...	...	54.1	0.2
0.80	0.60	...	9.4	...	...
1.00	0.60	18.5	...	94.4	0.2
1.20	0.60	...	0.3	...	0.5
2.00	0.60	38.8	0.6	28.8	0.6
3.00	0.60	80.1	...	3.0	0.6
4.00	0.60	78.8	...	1.8	...
6.00	0.60	75.4	...	...	...

TABLE VI.—HEMOLYSIS AT 25° OF HUMAN ERYTHROCYTES IN SOLUTIONS CONTAINING 0.6% SODIUM CHLORIDE AND VARIOUS PROPORTIONS OF AN ALKALOIDAL SALT

Alkaloidal Salt, %	NaCl, %	Ephedrine HCl	Ephedrine Sulfate	Atropine Sulfate	Homatropine HBr	Homatropine Methylbromide
0.00	0.60	0.4	0.6	0.2	0.0	0.3
0.50	0.60	0.0	...	1.6	...	0.0
0.80	0.60	...	0.7	...	...	...
1.00	0.60	0.0	0.5	7.4	0.6	0.3
1.50	0.60	0.0	0.3	...	0.0	0.0
2.00	0.60	0.3	0.8	0.0	24.0	0.0
3.00	0.60	0.5	0.0	0.6	43.5	0.2
4.00	0.60	0.2	...	0.5	27.5	0.0
6.00	0.60	0.2	...	...	7.0	...

ability data obtained from the use of one species to permeability relations involving a different species is not always advisable as has been pointed out earlier (3, 5, 7).

SUMMARY

1. Caffeine in concentrations ranging from 0.5 to 2.0 per cent failed to prevent hemolysis of rabbit and human erythrocytes even when used in the presence of 0.2 and 0.4 per cent sodium chloride. When tested in the presence of 0.6 per cent sodium chloride, caffeine caused no appreciable increase in hemolysis of erythrocytes.

2. Isotonic coefficients, i values, have been determined for several alkaloidal salts by use of the hemolytic method. Ephedrine hydrochloride and homatropine hydrobromide failed to show any appreciable prevention of hemolysis. Ephedrine sulfate and atropine sulfate gave unusually high i values. Homatropine methylbromide, on the other hand, gave a normal i value.

3. The effect of the addition of small proportions of sodium chloride on the isotonic coefficients obtained by the hemolytic method for various alkaloidal salts was studied. It was found that the addition of small proportions of electrolytes lowered the i values.

4. When tested with 0.6 per cent sodium chloride, ephedrine hydrochloride, ephedrine sulfate, and atropine sulfate appeared to cause an appreciable increase in hemolysis of rabbit erythrocytes. Atropine sulfate and homatropine hydrobromide appeared to cause an increase in erythrocyte fragility using human blood.

5. A comparison is given of results with rabbit and human blood.

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Fluorescence of Powdered Vegetable Drugs Under Ultraviolet Radiation*

By CHARLES J. KOKOSKI†, ROBERT J. KOKOSKI, and FRANK J. SLAMA

Numerous powdered vegetable drugs and some powdered animal drugs were examined under ultraviolet radiation after treatment with several reagents. The reagents were 1 *N* sodium hydroxide in methanol, 1 *N* sodium hydroxide in water, 1 *N* hydrochloric acid, nitric acid diluted with an equal volume of water, and sulfuric acid diluted with an equal volume of water. The characteristic fluorescence of each drug was observed and data pertaining to color and intensity were recorded.

MANY SUBSTANCES, including some powdered vegetable and animal drugs, exhibit fluorescence when exposed to radiation in the ultraviolet range, and frequently the fluorescence is sufficiently characteristic, both qualitatively and quantitatively, to be a useful tool in analysis. Progress in the analytical use of fluorescence has been reviewed (1).

Chase and Pratt cataloged the fluorescence response of 151 powdered vegetable drugs exposed to ultraviolet irradiation, and, on the basis of their study, developed a preliminary system of identification. They examined the drugs in three ways, i. e., (*a*) after mounting in a solution of nitrocellulose in amyl acetate, (*b*) after treating with 1 *N* sodium hydroxide in methanol, and (*c*) after mounting in a solution of nitrocellulose in amyl acetate following treatment with 1 *N* sodium hydroxide in methanol.

Since solvents and pH are capable of markedly influencing the fluorescent response of many substances, it seemed desirable to extend the work of Chase and Pratt. The reagents used in the present work and the conditions under which the drugs were examined are described in the following section.

EXPERIMENTAL

The source of ultraviolet light was a Hanovia Utility Model Ultraviolet Lamp; Hanovia Catalog No. 30600, equipped with a glass filter that transmitted in the range 3,600 to 3,700 Ångström units and absorbed the shorter ultraviolet radiations and visible light. All tests were made in a darkroom. Slides were placed at a distance of approximately six inches from the source of light. A black plate was used as a slide holder.

The following tests were performed on 131 powdered vegetable drugs and 2 powdered animal drugs in order to study their fluorescent properties:

I. Drug mounted in nitrocellulose in amyl acetate.[1] A sample of dry powdered drug was placed on a glass slide, affixed with nitrocellulose, allowed to dry, and then observed under the ultraviolet light.

II. Drug treated with 1*N* sodium hydroxide in methanol.

III. Drug treated with 1 *N* sodium hydroxide in methanol, dried, and mounted in nitrocellulose in amyl acetate.

IV. Drug treated with 1 *N* hydrochloric acid.

V. Drug treated with 1 *N* hydrochloric acid, dried, and mounted in nitrocellulose in amyl acetate.

VI. Drug treated with 1 *N* sodium hydroxide in water.

VII. Drug treated with 1 *N* sodium hydroxide in water, dried, and mounted in nitrocellulose in amyl acetate.

VIII. Drug treated with nitric acid diluted with an equal volume of water.

IX. Drug treated with sulfuric acid diluted with an equal volume of water.

For tests, II, IV, VI, VIII, and IX, a sample of dry powdered drug was placed on a slide, treated with several drops of the specified reagent, and observed immediately under the ultraviolet lamp. The color that develops in Test II must be observed within one minute because as the drug dries, due to the evaporation of methanol, the color may change.

For tests III, V, and VII a sample of dry powdered drug was placed on a slide, treated with several drops of the specified reagent, and allowed to dry completely. Then the dried specimen was mounted in nitrocellulose, allowed to dry, and observed under the ultraviolet lamp.

The colors emitted by the several drugs treated as outlined above were designated in terms of the three primary colors (red, yellow, and blue), the three secondary colors (orange, green, and purple), and brown, which is a mixture of the three primary colors (3). The term colorless was used to signify a complete absence of fluorescence: i. e., the drug was invisible and indistinguishable from the glass slide. Charts of hues, values, and chromas of colors were not used in this study because of the obvious difficulty in comparing emitted colors in a darkroom with reflected colors in visible light. To more clearly define some colors obtained, descriptive terms of shade, quality, and intensity of radiance were used.

RESULTS

The colors emitted when the 133 powdered drugs were subjected to the nine tests previously listed are classified in Table I, with letters signifying the

* Received February 7, 1958, from the Department of Pharmacognosy, University of Maryland, School of Pharmacy.

† Present address: George Washington University, School of Pharmacy, Washington 6, D. C.

[1] Testor's model airplane cement.

TABLE I.—FLUORESCENT PROPERTIES OF POWDERED VEGETABLE DRUGS STUDIED[a]

Vegetable Drug	Test I	Test II	Test III	Test IV	Test V	Test VI	Test VII	Test VIII	Test IX
Aconite	BG	BG 7	Fp 7	G 7	G	Gb	P	G 4	Bb
Agaricus	Gy	F	Fp	Y 7	Gf 2	Gf	Fy 7	Gy	P
Almond meal	Fp	Bg 7	PF	Bg	PF	BG 3	Fp	C	Gb 4 7
Althea	B 6 2	GB 2	Y	BG 7	G	G	Yg	GF 4 7	G
Anise	PF 2	B 2 6	Yf 2 6	Fr	Gf	G 7	Fy	Fg 7	Gf
Apocynum	Pr	BG 2 6	Gf	Pg	PF	G	Fp	C	Gb
Areca	Pr	BG 7	P 3	P	Pr	C	Pb 3	C	P 3
Asarum	YG 2	BG	Gf 2	Fg	Bf	G 3	Gb 7	G 3 7	Gb 7
Aspidium	Pr	Fg 3 7	G 7	F	Fg	C	Fg	C	F
Aspidosperma	Pr	Gf 2	Pr	B	BP	Gf	Pr	Bg	B 2
Belladonna leaf	OR 2 6	Gf 2	Gf 2	PF	Rp	Gb	Gf	C	Fg 7
Belladonna root	B	B	Gb	Bg	B 6	BG 6	Gb	Bg 3 7	Bg
Berberis	Yg 6	B 6	Yg 6	Yg 6	Yg 6	G 3 7	Yg 6	C	YG
Blue flag	Pr	Pg	Pg	P 3	Pr	G 3 8	P 3	C	YG
Buchu	OR 2	Gy 6	Fy	RF	Fy	Gf	Fy	F 4	Fg 7
Calendula	YO 7	BG	Yg 6	BG	OF 2	G 7	Gy 6	Bg	Gb
Canella	Y 2 6	G 7	Fy 2	Y	OY 2	G 3 7	Fy 2	Gf 7	Y 7
Cantharis	Fr	Fg	PF	Bg	Pg	G	P	Bg	Bg
Capsicum	Oy 6	Fg 1	Yf 2	Gf	Gf 7	Gf	Fg 2	BG	Gf
Caraway	Pr	Gf 1	Fg	Fr 7	Gf	Fg 7	Fg	F 4 7	Gf 7
Cardamom	Bg	B	Fg	Bg	Fy 2	G 3	Bf	Gb	Bg
Cascara sagrada	OF 2	Y	F	Fr	O 6	Og	Pr	FG	G
Cataria	R 2	R	G 2	Pr	R	Fr	F	Fr 3 7	Gf 7
Caulophyllum	G	B 6	Fy	Gf	Gf	Bg 3	Bf	Fg 3	Gb
Chenopodium	PF	G	Fg	Fg	BP	G 3	Gf	G 3 7	G 7
Chimaphila	Rp	G	Fg	P 3 7	Gf	G	Pf	C	Pf 7
Chondrus	Gf 2	Fr 2	P	Fg 2	P	Fg	F	Fg	Fg 2
Cinchona, red	P 3	F 3	Bg 3	P 3	Pr 3	F 4	Bg 3	B 6	B 6
Cimicifuga	Pb	G 3	Gb 3	Pg	B	G	Bg 3	Gb 3	Bg
Cinnamon, Saigon	OF 3	Gb 3	BG	Pr 3	F	G 3	BP 3	C	Pr 7
Cinnamon, Ceylon	Fr 3	G 3 7	BG 7	P	P	G 4 7	P 3	C	Pr
Clove	Fr	B 3	P	F 3 7	PR	C	Pb 3	C	F 7
Cocculus	Bf 2	Gb	F	Gf	Bf	G 3	F 3	G 3 7	G 7
Cochineal	Pr	Pg	Pr	Rb 3	O 6	R 3	C	F 3	PR 3
Colchicum root	YG 2	Bg 2	YGBF[b]	G 7	Gf	G 3 7	B	C	Gb
Colchicum seed	BG	BG	Bg	G 7	PF	G 3 7	Gb	C	G 7
Colombo	Y 6	Bg	Y 6	Fg	Y 6	BG 3	P	G 3 7	Gf
Conium	BP	YG	Pr	Bg	BP	Gf	P	Fg 3 7	Bg 7
Convallaria	Bg	BG 7	Pg	Bg	Gf	G 3 7	Gf	C	G 3 7
Coriander	P	BG 2	Yf	Br 2	PR	G 3	OF	F 3 7	Bg
Cornus	P	Pg 7	P	P	P	C	Pr	C	P
Corydalis	BG 2	B	P 2	Gf	BG 2	Bg 6	P	G 3	Gb 7
Cotton root bark	PR	G 3 7	Gb	P 3	PF	G 3 8	BP	C	P 3
Cubeb	Rp	BG	P 7	G 7	G 7	BG 7	BP	G 4 7	Gb
Curcuma	YG 6	O 6	YG 6	Yg	Gy 6	OF	Fr	Gf 3 7	O 7
Delphinium	B 2	Bg	By	Gy 7	Pg	G 7	P	Gf 3 7	G 7
Digitalis	PR 2	Fg 2	G	Pg 7	Or	Gf 3	Pg	C	Pg 7
Ergot	PF	Cf	OF	BG 3	Fg 7	G 3	Pr 2	G 3	Bg 3
Eriodictyon	PF	G	C	P	B	Gf 3 7	Rf 3	C	Pr 7
Eucalyptus	PG 2	Gf 7	Pg	PF	Gf 2	G 3 7	Fg	C	Pr
Fennel	P	Gb 2	P	Pf 7	Pg	G	Pf	C	Pf
Frangula	RO	YO 6	BP 3	OF	O	Gf 7	BP 3	Gf 7	OF
Gambir	F	G	C	R 3	Gf	G 3	Bg	C	Pr
Gelsemium	B 6	BG 2 6	BG	BP 6	Pb	Bg 6	BG	Pg 3	B 6
Gentian	YO	Yf 2	Of	PF	YG	G	OF	Cb 3	PF
Ginger	Gy 2 7	YG 6	O 2	YF	Bg	G	B 2	C	YF
Granatum	P	Gf 7	Pb	C	P	C	P 7	C	C
Grindelia	GB 6	Fy	OF	Pg	G	Fg 3	PF	Gf	Pg
Guaiac	Fg	B 6	C	Fg 3 7	P	B 6	P	C	G 8
Haematoxylon	Fg	F 3	BP	Pr 3	Gf	C	P	C	Pr 3
Hamamelis leaf	R 3	F	R	C	R	C	Pr	C	Pr
Hydrangea	PR 2	B 6	B 2	G 3 7	PR	B 6	Bg 2 6	Bg	G 2
Hydrastis	Yg 6	Gb	Y 6	Gf	YG	G 3 7	Pg	BG	Gf
Hyoscyamus	OR	Fg 2	Fp	Gp	PF	Fg 3	PF	G 3 7	BG 7
Ignatia	B 6	B	Y	BP	BP 6	F 3	FP	C	Pg
Inula	B 2	BG	Pr 2	Gp	B	Gf 3	Pf 7	Gf 3 7	Bg
Ipecac	B 2	BG	BG	BG	Bp	G 3	B	G 3	BG
Ipomea	BP	Bg	G	P	BP	G	Pf	G 3 7	P
Jalap	Bg 2 6	B 2	Gf	Bg	Pg	BG 3	Gf	G 3 7	Bg
Juglans	Pr	Gb 3 7	P	C	Pr	C	P	Pr 3 7	C
Kamala	P 3	C	P 3	C	C	C	Bg 7	C	C
Kava	Bg	G	Gb	Bg	BG	BG 2	B	C	YG
Krameria	Pr	P 6	P	Pr 3 7	Pr	Pr 3	P 3	C	P
Lappa	B 2	Gy 2	YG	Bg	BP	Gf 3	PF	Gb 3	Bg
Leptandra	BP	BG	P	Gf 3 7	P	G	P 3	C	G 7
Licorice	F	Gf	Br	Gf	Yf	G 3	Fg	G 3 7	Gf
Linseed	Bp	Bg	BP	Bg 7	BP	Gf	Pg	Fg 3 7	Bg 7
Lobelia	Or	Fg	Gf	Pf	OB	Gf 3	Gf	C	Pf
Lupulinum	Gf 2	G 6	Bf	B	Bg	Gf	Gf 2	Fg 3 7	Fg
Mace	F 1	Fg	Fp	Fy	BG	Fg	Pf	F 4 7	Fy
Matico	OR	OF	Pg	F	Gf 2	Gf	PR	FG 4 7	F
Matricaria	Gf 7	Bg	Gb	BG 7	Pf	Gb	Gf 2	Fg 7	Bg
Mustard, black	B 6	BG 6	Bg 2	Bg	BP	G	Bg 2 6	C	GB
Mustard, yellow	Gb	Gb	YO	Bg	P	G	Bg 6	C	Gb
Myrica	P	G 4 7	P	P 3	P	C	P 3 7	C	P
Myristica	Gp 7	FG	Pf	B	Fp	G 3 7	Pf 7	C	F
Nux vomica	P	G	BG	Pg 3 7	BP	Gf 4 7	P	C	Pg 7
Orange peel, bitter	Fb 1	YG 6	OF	Pg 7	Gf	G 3 7	Fy	BG 3	Bg
Orris, Florentine	Fg 1	Gy 2	Gf 2	P	F 2	Yg	Gb 6	F 3 7	P
Orris, Verona	Bp 2	Gy 2	Gf 2	P	F 2	G 6	Yg	F 3 7	P
Peppermint	PR	Fr	P 7	Pr 3 7	PR	C	P 7	C	Pr 3 7
Physostigma	B	B	BP	Bg 7	Pg	G 4 7	P 7	G 4 7	B
Phytolacca	Yf	Yf 2	Pf	Bg	Pf	Gb	Pf	BG	Bg
Pilocarpus	OR	Fr 2	Fr	Bf	Rp	Gf	PR	G 3	Gf
Pimenta	Pr	F	Pr	F 3 7	Pr	C	Pr	C	F 3 7
Pepper, black	B 3	B	BP	Gf	Bg	G 7	BP	C	Gf 7
Pulsatilla	Op	Y	G	BG 7	BP	Fg	PR	Fg 3 8	BG 7

TABLE I.—FLUORESCENT PROPERTIES OF POWDERED VEGETABLE DRUGS STUDIED (*Continued*)

Vegetable Drug	Test I	Test II	Test III	Test IV	Test V	Test VI	Test VII	Test VIII	Test IX
Pyrethrum flowers	Pf	Fg	Gy	Bf	P	F 3	Fp	Fg 3 7	Gb 7
Quassia	G 2	Gb 2	Gf 2	Bg	Bg	BG	Fg 2	G 3 7	Bg
Quercus	P	Bg	P 3	P	P	Gb	P 4	G 3 7	Bg
Red Saunders	Op	B 7	Y 7	P 4 8	Rp 6	BG 3	BP	C	R 3
Rhubarb	RO	G 7	C	Pr	OY 6	G 4 7	P	Fr	Fg 7
Sabadilla	P	Bg	Gb 2	G 4 7	P	G 3	P 3 7	C	G 4 7
Salvia	PR 2	Gf 7	P 3	P 7	Pr	Fg	PF	C	P 7
Sambucus	P 7	Gf	Fyg	Pg 7	P	Gf 3	Pr	F 4 8	Gf
Sanguinaria	Oy 5	Pb	O 6	Rp	O 6	G 3	YO 6	O	Pr
Sarsaparilla	Fg 1	Gf 1	Of	Gf	Pr 1	G	Gf 2	G 3 7	Gb
Sassafras	Pr 7	Pr	P 7	PF 7	P	Fr	P 7	C	PF
Scoparius	Op	Gf	Gf	Fg	F 3 7	Gf	PG	C	Gf
Scutellaria	Rp	Fg	BG 2	Bg 3 7	Pr 7	Gf	Pr 7	FG 3 7	FG 3 7
Senega	Bp 2	G 6	Yf 2	P	Bg 2	G 3	Gf	Fg 3 7	P 2 7
Senna, Alexandria	Fr	OF	F	Fr 3	OFg	Gf	F	R 3	F
Senna, Tinnevelly	Rf	OF	F 3	R 3	OF	Gf	F	R 3	R 3
Serpentaria	Gf 2	G 2	Gf 2	Gf	Fg 2	G 3	Gf	C	Fg
Spearmint	Fr	OF	Pg	PR 7	R	G 3 7	P 7	C	PR 7
Squill	B 2 6	GB 2	Bg 2	Bg	Pb	G 3 7	YG	Gb 3	Bg
Stavesacre	Pg	Gf 7	Pb	Gf 7	Pg	Gb 7	Pf	Gf 7	Gf 7
Stillingia	P 3 7	G 3 7	P 7	Pf 3 7	P	Gb 3	P	C	P 7
Stramonium	Rf	Fr	B	Fg 7	Pr	Fg	Fb	G 3 7	Fg
Strophanthus	B 2	B	BG	Pr 7	Bp	G	B	Gf 3 7	Pr 7
Sumbul	P	B 6	BG	Pg	P	Bg 6	P	BP 6	BP 6
Taraxacum	B 2 7	B 2	Bg	Bg	PG	Gf 3 7	Gf	G 3 7	Bg
Thyme	Pr	Gf 7	F	Pr	Bf	Gf 3 7	Fr	C	Pr
Trifolium	PR	Fg	Pf 2	Pf	P	Gf	PF	BG 3 7	Bf 7
Trigonella	Pr	Gf 2	Gf	Pf	Pg	Fg	P	BG 7	BG 3 7
Uva Ursi	Rf	F	Pr	Fr	Pr	R 3 8	Pr	Pr	Fr
Valerian	Bg 2 7	BG 2	YG 2	BG 7	B	G 3	Gf	G 3 7	BG 7
Veratrum viride	BP	B	B	P	P	Bg	Pg	G 3 7	P
Viburnum opulus	Pf 2	B	P	Pf	P	Gb 3	P 3 7	C	P
Viburnum prunifolium	P	Gb	Bg	P	P	G	P	C	P
White pine	P	B	Pg	P	P	BG 3	P	C	P
Wild cherry	P	B 6	Pf	P 6	BP	Bg 6	P	Bg	PB
Xanthoxylum	BP	B 6	BP	P 6	BP 6	BG 6	BP	G 6	Gb 2 6

[a] Key to letters and numbers used.

Predominant colors:		Modifying colors:		Quality of color:	
R	red	r	reddish	1	very light
O	orange	o	orangish	2	light
Y	yellow	y	yellowish	3	dark
G	green	g	greenish	4	very dark
B	blue	b	bluish	Degree of radiance	
P	purple	p	purplish	5	very bright
F	brown	f	brownish	6	bright
C	colorless			7	dull
				8	very dull

[b] Not a uniform color mixture.

various colors, and numbers signifying the various degrees of quality and/or intensity of radiance. Capital letters signify predominant colors and small letters signify modifying colors. For example; from the following code, (see footnote [a] Table I), a designation, Pb 3, would be dark bluish purple; while BG 3 7 would be dark, dull blue green, with blue and green equally predominant.

SUMMARY

The fluorescent properties of powdered vegetable drugs, treated with several reagents, were studied and the results are given in Table I. The characteristic fluorescent properties have shown themselves to be of valuable aid in the identification of the powdered vegetable drugs.

Some differences in observed fluorescence in this work as compared to the work of Chase and Pratt (2) may be due to the fact that drugs must be observed immediately after treatment, since colors may change upon evaporation of the solvents.

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The Pharmacology of Some Bis-Quaternary Ammonium Compounds*

By ZOLA P. HOROVITZ, EDWARD C. REIF, and JOSEPH P. BUCKLEY

Four experimental hypotensive agents [JB-520 (1,6-bis-N,N-dimethylamino-2-hexyne dimethobromide), JB-540 (1,6-bis-N,N-morpholino-2-hexyne dimethobromide), JB-549 (*beta*-[3-dimethylamino (propylamino)]-N-methylpiperidine dimethobromide), and JB-591 (*beta*-dimethylaminoethyl-N-methyl-pipecolinate dimethobromide)], and chlorisondamine dimethochloride were investigated for their pharmacological activities. All of the compounds blocked transmission in the cat superior cervical ganglion, blocked the hypertensive activity of DMPP in the rat, and dilated the mesenteric blood vessels of the rat. JB-520 and chlorisondamine depressed the auricular musculature of the perfused turtle heart, and JB-520 produced a negative inotropic effect on the isolated rabbit heart. All of the compounds increased the activity of the isolated rabbit ileum except JB-591, which was inactive. None of the compounds produced alterations in the blood pressure of the pithed rat.

SINCE Acheson and his co-workers (1, 2) described the relatively pure ganglionic blocking action of tetraethylammonium bromide, many investigators have described the pharmacologic actions of quaternary and bis-quaternary ammonium compounds. Crumpton, *et al.* (3), have reported that hexamethonium bromide decreased both cardiac output and coronary flow. Spoerel and Gowdey (4) have found that pentolinium produced a decrease in cardiac output in anesthetized dogs. Combes, *et al.* (5), have reported that the autonomic blockade by hexamethonium produced a fall in arterial blood pressure by reducing cardiac output and simultaneously interfering with compensatory peripheral arteriolar adjustments.

Paton and Zaimis (6) found that hexamethonium failed to produce a depressor effect in the pithed animal, indicating that the compound did not possess direct cardiac depressor or peripheral vascular activity.

Trapold and his colleagues (7, 8) have shown that chlorisondamine produced a decrease in mesenteric blood flow, in anesthetized dogs, with a comcomitant increase in mesenteric vascular resistance probably due to the release of an adrenergic substance.

Although the ganglioplegics have been reported to induce constipation and inhibit gastrointestinal activity in humans (9, 10) some investigators have reported that these compounds produced stimulatory effects on the stomach and intestines of experimental animals (11–13).

The hypotensive activity of a series of diquaternarized amino alcohol esters of piperidinecarboxylic acids and acetylenic bis-onium derivatives have previously been reported (14, 15). This present communication presents a more detailed pharmacologic investigation of four of the more potent hypotensive agents (Fig. 1) and chlorisondamine dimethochloride.[1]

EXPERIMENTAL PROCEDURES

Ganglionic Blocking Activity.—Adult cats of both sexes were anesthetized with an intraperitoneal injection of a mixture of urethan (250 mg./Kg.) and sodium pentobarbital (35 mg./Kg.). The preganglionic segment of the cervical sympathetic nerve was stimulated with a submaximal stimulus (determined for each amimal) using a Harvard stimulator at a frequency of 60 impulses per second for five seconds, and the effect on the nictitating membrane recorded. Three such stimuli were applied at five-minute intervals to determine an average normal response. A dose of one of the compounds was administered via a femoral vein and the submaximal stimulus applied at five-minute intervals. The average maximum per cent blockade was calculated at three logarithmically graded dose levels and the ED_{50} was estimated from the dose response curves plotted for each compound. Electrocardiographic recordings were obtained from lead II. Fourteen cats were used for this study.

The antagonism of the experimental compounds and chlorisondamine to the hypertensive activity of 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP),[2] a ganglionic stimulant (16, 17), was investigated in the rat. The animals were prepared as previously described (14) and a test dose of 200 mcg./Kg. of DMPP administered via the femoral vein. The hypotensive agent was administered ten

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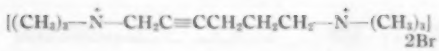
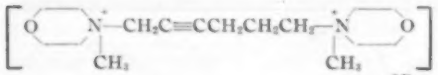
The experimental compounds were kindly supplied by Dr. H. L. Daiell, Scientific Director, Lakeside Laboratories, Inc.

[1] Kindly supplied by Dr. C. H. Sullivan, Ciba Pharmaceutical Products, Inc., Summit, N. J., as Ecolid chloride.

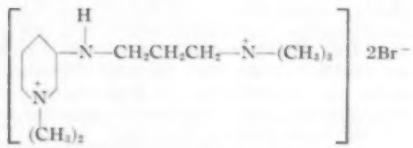
[2] Kindly supplied by Dr. R. F. Parcell, Parke Davis and Company, Detroit, Mich.



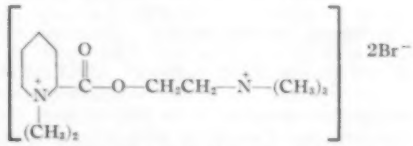
JB-520 1,6-*Bis*-N,N-dimethylamino-2-hexyne Dimethobromide



JB-540 1,6-*Bis*-N,N-morpholino-2-hexyne Dimethobromide



JB-549 Beta-3-dimethylamino propylamino-N-methylpiperidine Dimethobromide



JB-591 Beta-dimethylaminoethyl-N-methyl-pipecolinate Dimethobromide

Fig. 1.—Structures of the experimental hypotensive agents.

minutes after the hypertensive response and the test dose of DMPP repeated three minutes later. The mean per cent blockade to the test dose of DMPP was calculated at three logarithmically graded dose levels and the ED_{50} estimated by the de Beer graphic method (18).

Cardiac Effects.—The effects of the five compounds on the isolated rabbit heart were studied utilizing the method of Langendorf (19) as modified by Anderson and Craver (20). One-ml. doses of the compounds in dilutions of 1:100 to 1:1,000 were injected into the perfusion system just above the heart and washed in with 1 ml. of perfusate.

The compounds were investigated in the *in situ* turtle heart perfused with turtle Ringer's solution. Heart levers were prepared to record contractions of the ventricle, right auricle, and sinus venosus on a slowly moving kymograph. One-half-ml. doses of varying concentrations of the compounds were administered into the venous cannula.

Peripheral Vascular Effects.—The rat meso-appendix preparation (21) was employed in studying the comparative effects of the drugs on the mesenteric blood vessels. Wistar rats, weighing between 100 and 250 Gm. were anesthetized with urethan, 1.25 Gm./Kg. i. p. The animal was affixed, ventral side up, to a plexiglass platform. The meso-appendix was exposed and spread into proper position and the platform placed on the stage of the microscope. The exposed tissue was moistened with frequent applications of normal saline solution warmed to 38°. The approximate hypotensive $dose_{50}$ of each compound (14, 15, 25) was administered via the femoral vein. Photographs of a single field (100 × magnification) were taken prior to and at five minute intervals after drug administration. Evaluation was made by projecting the 35 mm. slide to a definite magnification and grading the alteration in the appearance of the vascular field from 0 (no change) to +3 (maximum increase in the vascular bed). The diameter of one of the magnified arterioles in the field was measured in mm. units prior to and after drug administration. A total of 19 rats were utilized for this study.

The compounds were also investigated in the pithed rat (22). Blood pressures were recorded from the left common carotid artery via a small bore mercury manometer and artificial respiration accomplished by means of a Phipps and Bird Variphase valve attached to a source of compressed air. The approximate hypotensive $dose_{50}$ of each compound was administered via a femoral vein. A minimum of five animals was employed for each compound.

Intestinal Studies (in vitro).—The compounds were studied on the isolated rabbit ileum as per Magnus (23). Strips of rabbit ileum, approximately 2 cm. in length, were mounted in a 70-ml. bath containing aerated Tyrode's solution maintained at 37.5°. The compounds were administered to the bath to produce dilutions from 1:70,000 to 1:70,000,000.

RESULTS AND DISCUSSION

Ganglionic Blocking Activity.—The dose producing a 50% ganglionic blockade (ED_{50}), in the cat, was determined for each of the five compounds. It was found that 0.5 mg./Kg. of JB-520, 2.5 mg./Kg. of JB-540, 1.0 mg./Kg. of JB-549, 1.0 mg./Kg. of JB-591, and 0.25 mg./Kg. of chlorisondamine dimethochloride were necessary to produce 50% blockade. A typical response to JB-591 can be seen in Fig. 2. Postganglionic stimulation elicited effects similar to pretreatment responses.

The dose producing a 50% blockade of the DMPP hypertensive response in the rat was also determined for each compound and these doses compared to the hypotensive $dose_{50}$ in the rat (Table I). Chlorisondamine dimethochloride exerted the greatest antagonism to the hypertensive activity of DMPP in

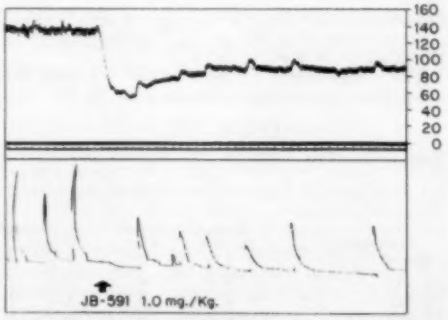


Fig. 2.—The effect of JB-591, 1.0 mg./Kg., i. v., on the cat nictitating membrane preparation. Upper tracing, effect on blood pressure; lower tracing, nictitating membrane response.

TABLE I.—THE EFFECTS OF CERTAIN BIS-QUATERNARY AMMONIUM COMPOUNDS AGAINST THE HYPERTENSIVE ACTIVITY OF 200 MCG./KG. OF DMPP IN THE RAT

Compound	No. of Rats	ED_{50} ± S.D., mg./Kg.	Hypotensive $Dose_{50}$[a]	% of Hypotensive $Dose_{50}$
JB-520	9	7.8 ± 1.6	5.0	156
JB-540	9	6.8 ± 1.5	10.0	68
JB-549	9	9.3 ± 1.4	5.0	186
JB-591	12	4.7 ± 1.3	25.0	19
Chlorisondamine	12	0.17 ± 0.15	0.5	34

[a] As reported (14, 15, 25).

the rat. It is also interesting to note that chlorisondamine was the most potent ganglionic blocking compound when tested in the cat ganglion-nictitating membrane preparation. The dose of JB-591 producing 50% blockade to the hypertensive response of DMPP was approximately 1/5 the hypotensive $dose_{50}$ and was the smallest fraction of the hypotensive dose of any of the compounds tested. Both JB-520 and 549 required doses larger than their respective hypotensive $dose_{50}$ to produce a 50% blockade to DMPP.

Cardiac Effects.—JB-520 was the only compound to elicit any action on the isolated rabbit heart. It produced a slight decrease in coronary flow, in all concentrations, lasting for approximately twenty minutes and accompanied by a negative inotropic effect. JB-520 produced both negative inotropic and chronotropic effects in the perfused turtle heart in dilutions of 1:500 to 1:1,000 persisting from one to five minutes (Fig. 3). These effects were most evident on auricular musculature. One-half-ml. doses of JB-520 in concentrations between 1:100 and 1:200 produced cardiac arrest for periods up to twelve minutes. The effects of JB-520 on the turtle heart were blocked by 1-mg. doses of atropine sulfate indicating a possible parasympathomimetic action.

The action of JB-520 on the perfused turtle heart corresponds favorably with the effects observed in the isolated rabbit heart. These are opposite to the effects of hexamethonium on the cat papillary muscle preparation as reported by Lee and Shideman (24). Chlorisondamine dimethochloride produced depression of the auricular musculature of the turtle in concentrations of 1:100 to 1:200 without affecting the ventricle or sinus venosus.

The experimental compounds did not alter the normal electrocardiographic patterns in the cat.

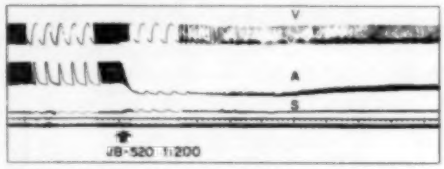


Fig. 3.—The effect of JB-520 on the perfused turtle heart. A = right auricle, S = sinus venosus, V = ventricle.

JB-549, 2.5 mg./Kg., and JB-591, 5.0 mg./Kg., decreased cardiac rate approximately 50% one hour after drug administration. JB-540, 5.0 mg./Kg., and JB-520, 1.0 mg./Kg., produced a decrease in cardiac rate of approximately 40% one hour after drug administration. Bradycardia persisted even after the blood pressure returned to normal. The decrease in cardiac rate in the anesthetized cat may possibly be due to the low vagal tone in the anesthetized animal and a blockade of sympathetic activity by the ganglioplegic agents.

Peripheral Vascular Activity.—The effects of the compounds on the mesenteric blood vessels of the rat are summarized in Table II. All of the compounds produced vasodilatory effects, with JB-540 and JB-549 appearing to be slightly more active than the other three compounds. The changes in the arteriolar diameter, although generally slight, correlated with the general appearance of the vascular field.

The compounds did not alter the blood pressure of the pithed rat. A slight transient hypotensive response was observed after the administration of each compound, however this appears to be due to injection as administration of equal volumes of saline prior to the drugs elicited similar responses. The inactivity of the compounds in the pithed preparation indicates the absence of direct peripheral vascular and direct cardiac depressor activities. These results are similar to the observations with hexamethonium by Paton and Zaimis (6).

Intestinal Activity.—All of the compounds except JB-591 induced increases in the contractions of the isolated rabbit ileum. JB-549 was the most potent compound as dilutions of 1:70,000 to 1:7,000,000 produced sharp contractions of the ileum. Dilutions of JB-520 from 1:70,000 to 1:700,000 also elicited sharp contractions, whereas JB-540 and chlorisondamine were ineffective in concentrations between 1:700,000 and 1:7,000,000. The latter two compounds produced slight increases in the amplitude of contractions in 1:70,000 dilutions.

TABLE II.—THE EFFECTS OF CERTAIN BIS-QUATERNARY AMMONIUM COMPOUNDS ON THE VASCULAR BED OF THE RAT MESO-APPENDIX

Compound	Dose, mg./Kg.	No. of Rats	Mean Change in Field[a] 5 Min.	10 Min.	15 Min.	Mean Diameter of Arteriole in Units Normal	5 Min.	10 Min.
JB-520	5.0	4	+1	+1	+1	4.8	5.3	5.0
JB-540	10.0	4	+2	+1½	..	7.8	9.0	9.0
JB-549	5.0	4	+2	+2	+2	6.5	7.3	..
JB-591	25.0	4	+1	+1½	..	4.8	6.0	6.0
Chlorisondamine	0.5	3	+1	+1½	..	10.3	10.7	12.0

[a] 0—no change, +1—slight increase, +2—moderate increase, +3—maximum increase.

SUMMARY

1. All of the compounds produced blockade of preganglionic stimulation of the cervical sympathetic nerve in the anesthetized cat as demonstrated by the ability to block nictitating membrane retraction. Chlorisondamine dimethochloride was the most potent.

2. All of the compounds were antagonistic to the hypertensive effects of 1,1-dimethyl-4-phenylpiperazinium iodide in the rat. Chlorisondamine dimethochloride was the most potent of the compounds studied. JB-591 was the most potent of the experimental compounds.

3. JB-520 was the only compound to demonstrate any activity on the isolated rabbit heart by producing a slight decrease in coronary flow and a negative inotropic action.

4. JB-520 and chlorisondamine dimethochloride produced auricular depression in the perfused turtle heart.

5. All of the compounds produced vasodilatory effects on the vascular bed of the rat meso-appendix.

6. JB-520, JB-540, JB-549, and chlorisondamine stimulated the isolated rabbit ileum. JB-591 produced no apparent changes in the activity of the isolated intestinal strips.

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The Culture of *Claviceps purpurea* IV. Lipogenesis*

By JAMES E. DUSENBERRY† and ARTHUR E. SCHWARTING

The relationship of time and variation in media and the resulting effect on lipid formation in submerged culture are discussed. The data indicate that the type of reaction vessel, together with the carbon source, limited growth weight and the formation of lipid material. A gradual increase in the initial concentration of carbon from two to eight per cent, showed a gradual increase in lipid concentration. Certain additives produced a variety of effects on the accumulation of lipids.

SAPROPHYTIC CULTURE studies of *Claviceps purpurea* (Fries) Tulasne have ordinarily been directed to understandings concerning growth or alkaloid production. Ergot sclerotia contain considerable lipid substance and notable studies have been made on the composition of this product (1–3).

Preliminary studies indicated a comparatively low lipid accumulation in the mycelium of submerged grown ergot. This study was undertaken to determine certain conditions which governed lipid accumulation in ergot in saprophytic culture.

EXPERIMENTAL

The culture of ergot, designated RCT, used in these experiments, was obtained from mature rye sclerotia. The initial growth, from sclerotia, was subcultured to agar slants of a mannitol-casein hydrolysate-mineral salts (4) medium (see Media) and was refrigerated. All subsequent subcultures were made from these slants to liquid media, of the same composition, in 500-cc. Erlenmeyer flasks. Aliquot portions of six day old submerged grown mycelium were used for all inoculations.

In the studies on lipid accumulation, ergot was

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grown in carboys, in a Pfaudler chemical reactor, adapted for use as a fermentation vessel, and in 500-cc. Erlenmeyer flasks. All fermentations were conducted at 22.5° ± 1°.

Twelve-liter, wide-mouth Pyrex carboys were each fitted with rubber stoppers, through which a 3.75 x 20-cm. glass tube was inserted. Fifteen hundred cc. of medium was added and a cotton stopper was inserted in the glass tube. Each vessel of sterilized and cooled medium was inoculated with 100 cc. of inoculum. Aeration was accomplished by rotary agitation at 57 rotations per minute.

A model 801, 5-gallon Pfaudler reactor was modified to provide sterile oxygen intake and exhaust, an inoculation port, and temperature control. Sixteen liters of media was sterilized, in the reactor, and after cooling, was inoculated with 400 cc. of inoculum. Agitation was accomplished with the anchor stirrer, 150 r. p. m., together with a baffle rod and an air sparger. Oxygen was introduced, through sterile cotton, at a rate of 8 L. per hour.

The Erlenmeyer flasks, containing 100 cc. of sterilized media, were inoculated with 0.8 cc. of mycelium, transferred from the inoculum with a fungal cup. The flasks were placed on a reciprocal shaker at 114 excursions per minute.

Media.—A basic nutrient solution composed of $MgSO_4 \cdot 7H_2O$, 1.25; KH_2PO_4, 2.5; and casein hydrolysate 10.0 Gm./L. distilled water was used in these investigations. Twenty Gm./L., as well as higher concentrations of the carbon source, mannitol or dextrose, was used. Calcium pantothenate, zinc sulfate, ergot oil, or calcium carbonate was added to this basic medium in certain experiments.

Mycelium Collection and Extraction.—When collected, mycelium was obtained by filtration with Büchner funnels. The mycelial mat was washed with distilled water and all excess water was expressed. The mat was transferred to deep porcelain dishes which were placed in a dry ice–alcohol mixture until the mycelium was frozen. The mat was freeze-dried and was stored in a desiccator, over calcium chloride, until it was prepared for extraction. The dried product was reduced to a powdery product in a Waring Blendor and was further comminuted by grinding in a ball mill for forty-eight hours. The weighed product was extracted for forty-eight hours in a Soxhlet apparatus, using petroleum ether (b. p. 40–60°). Additional extraction with petroleum ether or additional extraction after digestion of the extracted tissue with hydrochloric acid did not appreciably raise the oil yield.

EXPERIMENTAL RESULTS

The growth weight and lipid production in the carboys was comparable to that of the Erlenmeyer flasks with each of the two carbon sources. The Pfaudler reactor was a less efficient system for growth and for lipid production. The quantitative data reported in these experiments were derived from growth studies in the carboys.

The data of Table I are representative of a series of trials relating growth and lipid accumulation for the two carbon sources. It was further determined that neither a decrease nor increase in the casein hydrolysate concentration in a basic medium with 2% mannitol caused a significant change in growth, growth weight, or lipid production. Using 1.0% each of mannitol and dextrose, in a single medium, an intermediate growth weight and lipid content was obtained when compared to the data of Table I for the two separate sugars. Lipid accumulation was obviously more rapid and greater in the basic medium containing glucose, and it was also apparent that the lipid reserve diminished rapidly during the latter period of a twenty day fermentation time.

A number of factors were found which contributed to reduced or slow growth together with an increase in absolute lipid accumulation. Calcium carbonate, 1.33%, although reducing growth only slightly, favored lipid accumulation. Increasing the carbon concentration, Table II, caused a slower growth rate and markedly increased the absolute lipid accumulation. The highest concentration of glucose, 10%, caused a reduction in lipid accumulation and the tissue produced in this medium and in media containing 8% glucose was predominantly conidia.

Pantothenic acid has been shown to increase lipid synthesis in yeasts (5, 6). Ergot grown in a basic medium with 2% mannitol for 168 hours, and containing calcium pantothenate 10 μg./cc., was not significantly influenced by the vitamin either in growth or in absolute lipid accumulation.

Zinc, 9.0 mg./L., as zinc sulfate caused a slight reduction in growth and caused a slight percentage increase in lipid accumulation. Absolute lipid accumulation in 168 hours was 0.41 Gm. in media containing 2% mannitol and 0.49 in media containing 2% dextrose.

Evidence obtained throughout the investigation demonstrated that the absolute amount of lipid began to decrease when the growth rate declined. Ergot oil, filter sterilized, 0.03% in basic media containing 2% mannitol, caused a marked increase (45%) in growth, in 168 hours. The absolute lipid accumulation was also in excess (34%) of the theoretical.

TABLE I.—GROWTH AND LIPID ACCUMULATION IN ERGOT

Fermentation Conditions		Yield		
Carbohydrate, Concn., %	Growth Time, hr.	Growth Wt., Gm.	Oil Content, %	Absolute, Oil Content, Gm.
Mannitol, 2	120	15.6	1.20	0.187
	240	20.5	1.91	0.392
	360	17.0	1.82	0.309
	480	16.3	1.30	0.212
Glucose, 2	120	22.2	2.39	0.531
	240	25.2	2.30	0.580
	360	15.2	1.84	0.280
	480	10.2	1.24	0.126

TABLE II.—CARBOHYDRATE CONCENTRATION, GROWTH, AND LIPID ACCUMULATION

Fermentation		Yield	
Mannitol Concn., %	Time, Hr.	Growth, Gm.	Absolute Oil Content, Gm.
4[a]	168	22.4	0.72
6[a]	216	24.1	1.06
8[a]	288	23.4	2.21
10[a]	360	29.3	0.97

[a] $CaCO_3$, 1.33%.

CHEMICAL AND PHYSICAL PROPERTIES OF THE LIPID

The oil, largely obtained from mycelium grown in the Pfaudler reactor, was a viscous yellow to occasionally brown-red liquid usually containing suspended material and contained an average of 8.76% of unsaponifiable matter, about 50% of which was soluble in hot ethanol.

The alcohol-soluble portion of the unsaponifiable matter was converted to the digitonide and the sterols were regenerated with dry pyridine. The product, 0.4628 Gm., from 12.01 Gm. of oil, melted at 157–158° (Kofler). Repeated crystallization from ethanol and ethyl acetate gave a product having a melting point of 160–161° (Kofler) and a specific rotation of $[\alpha]_D^{25}$ − 129 ($CHCl_3$). The acetate, recrystallized from ethanol melted at 178–179° (Kofler). These data together with R_f values obtained on Quilon[1] impregnated paper propound the existence of ergosterol as the major sterol in the oil.

The alcohol-insoluble unsaponifiable material, 0.5206 Gm. from the 12.01 Gm. of oil, was chromatographed on a 0.9 x 15-cm. column of alumina, using petroleum ether (40–60°) as the wash liquid. The eluate yielded 0.2267 Gm. of white wax-like solid m. p. 58–60°; n_D^{25} 1.6874. The substance was insoluble in concentrated sulfuric acid and in fuming sulfuric acid.

DISCUSSION AND SUMMARY

The percentage of lipid material did not approach that accumulated by the same organism growing in its natural parasitic form.

Neither an increase nor decrease in the casein hydrolysate concentration in a basic medium with 2% mannitol caused a significant change in growth, weight, or lipid production.

Glucose was shown to be superior to mannitol as the carbon source for lipid accumulation. And there was a loss of the lipid reserve during prolonged fermentation periods. Increasing the carbon concentration caused a slower growth and increased the lipid accumulation. The highest concentration of glucose, 10% caused a reduction in lipid accumulation. Zinc caused some increase in the lipid content while the addition of ergot oil caused a rather marked increase in growth weight and absolute lipid accumulation.

The viscous yellow to red-brown oil contained 8.76% of unsaponifiable matter. The sterol portion was predominately ergosterol. The properties of the alcohol-insoluble unsaponifiable material indicated that it was a hydrocarbon.

[1] du Pont product information bulletin, "Quilon," January, 1950. Quilon kindly supplied by E. I. du Pont de Nemours and Co., Inc.

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Degradation of Epinephrine Induced by Bisulfite*

By LOUIS C. SCHROETER, TAKERU HIGUCHI, and EDWARD E. SCHULER

Degradation of epinephrine solutions stored in an oxygen free atmosphere in the presence of as little as 0.1 per cent bisulfite occurs at faster rates than in complete absence of bisulfite. Loss of epinephrine chemical activity as determined by the triacetyl derivative procedure occurs with concomitant bisulfite loss. Change in optical rotation and in chemical activity upon storage at 80° in sealed, evacuated ampuls has been studied with various buffer systems at different pH values and with different concentrations of reactants. An inactive degradation product has been isolated and partly characterized.

ALTHOUGH many commercial liquid formulations of epinephrine contain bisulfite as an antioxidant, evidence is presented in this communication which strongly suggests that this additive may, under certain conditions, actually accelerate the loss of the drug from solution. The phenomenon has been observed so far only in essentially neutral systems which would not undergo rapid proton catalyzed racemization and were stored under oxygen free atmosphere. These conditions exist, however, in many parenteral preparations of this drug. Since the possibility of this type of deteriorative reaction has not been previously realized and because of the rather un-

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usual nature of the mechanism involved, a short report on the general characteristics of the reaction is presented at this time.

The occurrence of this type of nonoxidative deterioration of epinephrine solutions was first suspected during accelerated testing of commercial preparations. Solutions of the drug in sealed ampuls under nitrogen showed progressive loss in activity by both physiological assay (1) and by chemical assay not totally attributable to racemization. The observed rate of loss appeared to be dependent in part on the bisulfite concentration, the rate of degradation being much faster in presence of as little as 0.1 per cent sodium bisulfite than its total absence.

In the present study, a more formal investigation of the influence of the substrate concentration, bisulfite concentration, and pH on the reaction rate has been carried out. The relationship of this reaction to the racemization reaction reported by Kisbye (2) has also been explored and compared with the proton-catalyzed racemization reaction previously reported by this laboratory (3). An inactive transformation product from epinephrine has been isolated and partly characterized.

EXPERIMENTAL

Epinephrine solutions of varying concentrations in acetate, citrate, and phosphate buffers of varying pH and ionic strength were filled into hard glass ampuls. These solutions served as blanks. Identical solutions were prepared containing, in addition, varying concentrations of sodium bisulfite. The pH of these solutions was adjusted to that of the appropriate blank and then filled into ampuls. The filled ampuls were evacuated to remove atmospheric oxygen and sealed under vacuum. The solutions were stored in a thermostated water bath at 80°.

Sampling was performed by periodically removing ampuls from the thermostat and chilling in an ice water bath to quench the reaction. Prior to analysis, the chilled ampuls were thermostated briefly at 25° to prevent volume errors due to temperature effects. The pH of each solution stored at elevated temperatures was determined. Optical activity of the solutions at 25° was determined with a Zeiss Winkel polarimeter using filtered sodium light (589 mμ); 0.5, 1.0, or 2.0 dm. micro tubes were employed. Blank solutions which did not contain bisulfite tended to develop color of such intensity that accurate polarimetry was very difficult. Measurements were obtained by decolorizing blank solutions with small amounts of activated charcoal, the procedure was checked against known concentrations of epinephrine in the presence of adrenochromes.

The ultraviolet spectrum from 220 to 400 mμ of each sample was determined using the Cary Model 11 Recording Spectrophotometer. This procedure was followed to determine if a spectral shift or change in absorbance of the epinephrine solutions took place. Chemical activity of epinephrine was determined by a modification (4) of the triacetyl derivative procedure introduced by Welsh (5) in which epinephrine in dilute aqueous solution is quantitatively converted into its O^3, O^4, N-triacetyl derivative. Samples representing 5 to 10 mg. epinephrine were obtained by making proper dilutions of the reactant solutions. Bisulfite present in samples was destroyed with iodine T.S. prior to acetylation since Welsh reported that it interfered by catalytically accelerating the hydrolysis of acetic anhydride (6,7). Acetylation was effected by the addition of accurately measured quantities of acetic anhydride and sodium bicarbonate to the mechanically stirred epinephrine solution.

The triacetyl derivative was isolated from the acetylated solution by partition chromatography. The acetylated solution was slurried with Celite 545 and isooctane-chloroform solution and packed on top of a pH 5 buffered Celite column. The column was then developed with chloroform. The ultraviolet spectrum and absorbance of the chloroform eluate containing triacetylepinephrine was determined spectrophotometrically. The bisulfite content of each sample was determined by iodometric titration, the milliequivalents of iodine required to titrate solutions containing only bisulfite and buffer being subtracted from the milliequivalents used to titrate the sample.

OBSERVATIONS

Typical degradative behavior of epinephrine in solution in presence of bisulfite is shown in Figs. 1–3. In obtaining these plots the drug concentration was determined by the triacetylation procedure and 0.1% concentration of epinephrine and bisulfite, when present, were used. As could be seen, the chemical assay shows no loss of the active component in absence of bisulfite but a substantial breakdown in its presence. More detailed behavior of the reaction is discussed in the following sections.

Order of the Loss Reaction with Respect to Epinephrine.—Originally a considerable amount of information was accumulated which suggested that the rate of loss of epinephrine from these systems was first order with respect to the drug. The loss

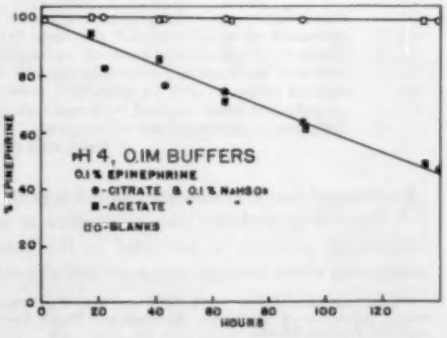

Fig. 1.—Per cent epinephrine remaining in 0.1% (0.0055 molar) solutions containing 0.1% sodium bisulfite (0.0096 molar) buffered at pH 4.0 with 0.1 molar acetate or citrate. The solutions were stored in sealed, evacuated ampuls at 80°.

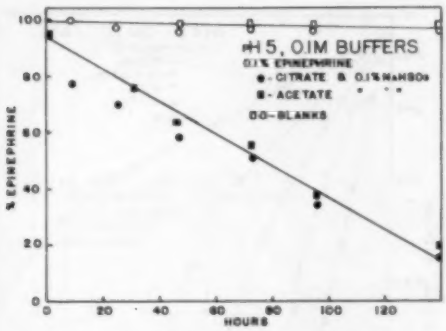

Fig. 2.—Per cent epinephrine remaining in 0.1% (0.0055 molar) solutions containing 0.1% sodium bisulfite (0.0096 molar) buffered at pH 5.0 with 0.1 molar acetate or citrate. The solutions were stored in sealed, evacuated ampuls at 80°.

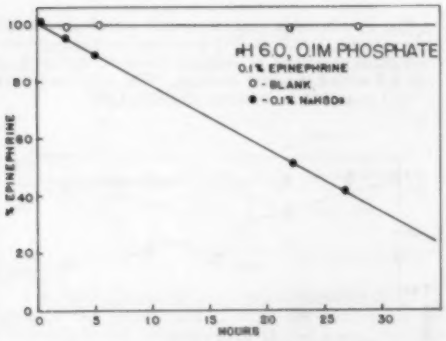

Fig. 3.—Per cent epinephrine remaining in 0.1% (0.0055 molar) solutions containing 0.1% sodium bisulfite (0.0096 molar) buffered at pH 6.0 with 0.1 molar phosphate. The solutions were stored in sealed, evacuated ampuls at 80°.

curves for example seemed to be linear with respect to time as shown in Figs. 1–3. Another significant evidence was that proportionally much greater loss was experienced with 0.1% solutions than with 1.0% solutions in pilot plant preparations. Situations of this type are normally found only with zero order reactions.

Further study of the reaction system gave evidence, however, to the belief that the loss rate was basically directly dependent on the drug concentration. In presence of higher concentration of sodium bisulfite, the loss curve assumed the typical first order logarithmic decay form, Fig. 10. The apparently greater proportionate loss of 0.1% solutions as compared to 1.0% solutions in commercial lots was found to be explicable in part as being due to rapid exhaustion of antioxidant in the more concentrated systems. Both contained the same amount (0.1%) of bisulfite.

Role of Bisulfite.—Since the loss of epinephrine occurred in absence of free oxygen, it was initially thought that bisulfite may possibly play only a catalytic role for the reaction, but this was found to be not true. Determination of the bisulfite concentration in the degrading system showed a steady decrease. This is evident in Fig. 4 where experimentally determined concentrations of the sulfur compound are shown for various reaction times. The data indicate that only one molecular equivalent disappears for each molecule of epinephrine lost from the system.

The influence of bisulfite concentration on the rate of disappearance of the drug is apparent in Figs. 5, 10, and 11 where residual concentrations of epinephrine as determined by the triacetyl method have been plotted against reaction time in presence of varying bisulfite concentration. At higher antioxidant concentration, as previously mentioned, the rate of drug loss appears to be strictly first order. At lower levels this does not seem to be true. Although the reaction rate is increased by an increase in bisulfite concentration, a direct proportionality does not seem to exist between the two.

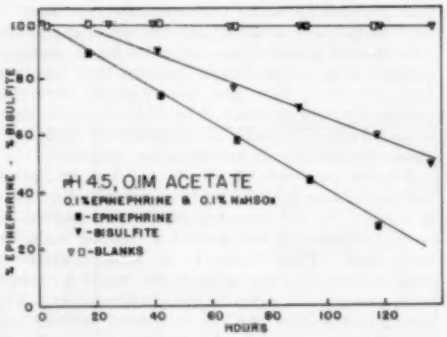

Fig. 4.—Per cent epinephrine and per cent bisulfite remaining in 0.1% (0.0055 molar) epinephrine solutions containing 0.1% sodium bisulfite (0.0096 molar) buffered at pH 4.5 with 0.1 molar acetate. The solutions were stored in sealed, evacuated ampuls at 80°.

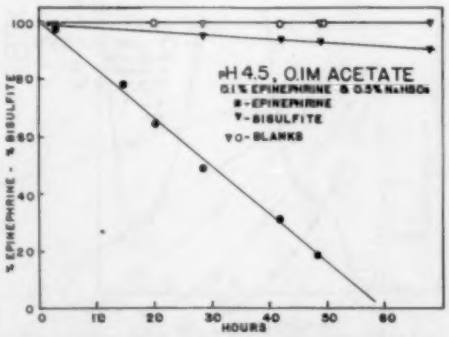

Fig. 5.—Per cent epinephrine and per cent bisulfite remaining in 0.1% (0.0055 molar) epinephrine solutions containing 0.5% sodium bisulfite (0.048 molar) buffered at pH 4.5 with 0.1 molar acetate. The solutions were stored in sealed, evacuated ampuls at 80°.

Influence of pH.—No clear-cut interrelationship between the overall degradation rate and hydrogen ion concentration could be deduced from such data as were obtained. Under comparable conditions the loss rate appears to increase with rise in pH as shown in Figs. 1–3. The times necessary to reach 50% loss under conditions of study were approximately as follows:

pH	Time, hr.
4.0	140
5.0	70
6.0	23

Further studies are necessary to determine the mathematical relationship between pH and rate of loss.

Relation to Loss in Optical Activity.—A rather complex relationship seems to exist between racemization of the optically active drug and the bisulfite reaction. This is particularly evident in Fig. 10 where the degrading system has been followed by both polarimetric and chemical analyses. As with previous runs, the total epinephrine concentration shows no detectable loss even after forty hours. The optical activity, on the other hand, shows a decided drop. This would indicate that the drug had racemized but had not changed otherwise chemically. In presence of bisulfite, however, there is no difference between the polarimetric results and those obtained by the triacetylation procedure.

Further evidence of accelerated loss in optical activity induced by bisulfite is presented in Figs. 6 and 7. In the first the results are plotted directly, whereas in the second a semilog scale has been used. There seems to be some evidence in these curves that the order of the reaction changes from pseudo zero order to pseudo first order. Figures 8 and 9 show the loss in optical activity and the chemical activity of solutions in which epinephrine is present in large excess (0.11 and 0.22 molar) compared to bisulfite (0.0096 molar). The solutions were buffered at pH 4.5 with 0.5 molar acetate and stored at 80° in sealed, evacuated ampuls up to one hundred and forty hours. The chemical activity of the solutions showed no significant change during the test period indicating that the chemical loss is contingent upon the presence of bisulfite in near stoichiometric amounts. The loss of optical activity of blank solutions and solutions stored with bisulfite was essentially the same when epinephrine was present in large excess.

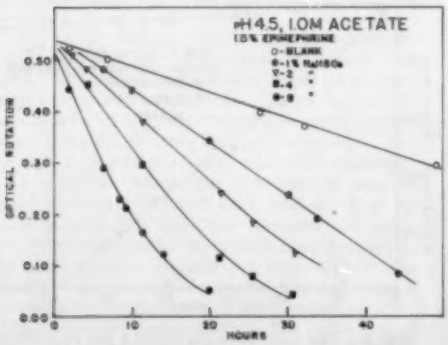

Fig. 6.—Optical rotation of 1.0% epinephrine (0.055 molar) solutions containing various concentrations of sodium bisulfite buffered at pH 4.5 with 1.0 molar acetate. The solutions were stored in sealed, evacuated ampuls at 80°.

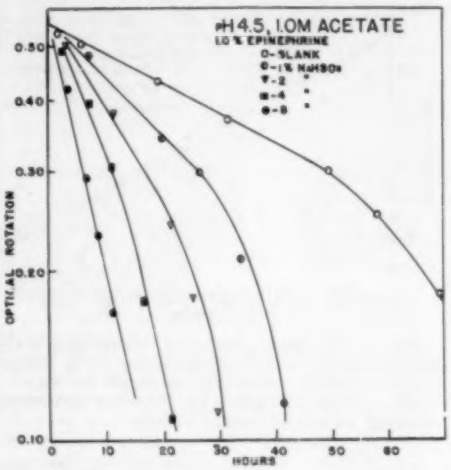

Fig. 7.—Semilog plot of optical rotation of 1.0% epinephrine (0.055 molar) solutions containing various concentrations of sodium bisulfite buffered at pH 4.5 with 1.0 molar acetate. The solutions were stored in sealed, evacuated ampuls at 80°.

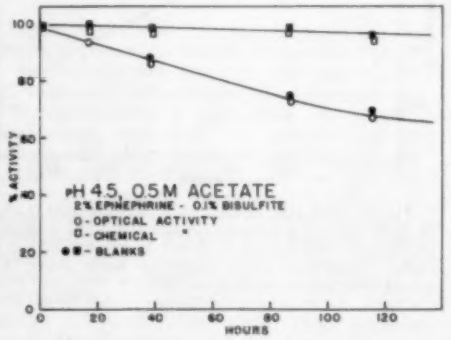

Fig. 8.—Optical and chemical activity of solutions containing 2% epinephrine (0.11 molar) and 0.1% sodium bisulfite (0.0096 molar) buffered at pH 4.5 with 0.5 molar acetate and stored at 80° in sealed, evacuated ampuls.

Isolation of the Bisulfite Addition Compound.—Partition columns which gave low recoveries of epinephrine as the chloroform soluble triacetyl derivative were further eluted with solvents of increasing polarity. Methanol-water elution yielded an acetylated product having nearly the same ultraviolet spectrum as triacetylepinephrine but which differed markedly in solubility behavior. It was insoluble in chloroform and benzene, but readily soluble in water or methanol. Upon evaporation

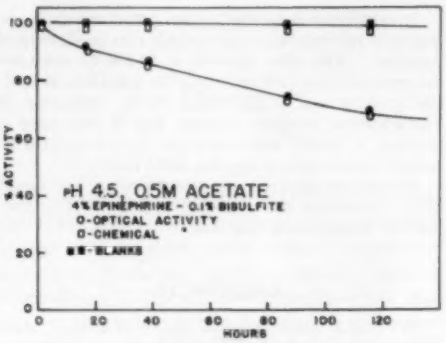

Fig. 9.—Optical and chemical activity of solutions containing 4% epinephrine (0.22 molar) and 0.1% sodium bisulfite (0.0096 molar) buffered at pH 4.5 with 0.5 molar acetate and stored at 80° in sealed, evacuated ampuls.

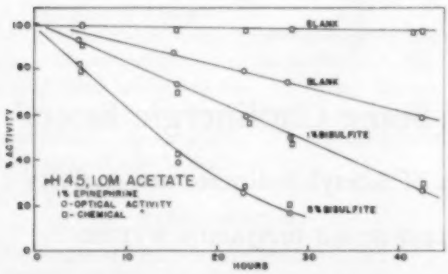

Fig. 10.—Chemical and optical activity of 1.0% epinephrine (0.055 molar) solutions containing bisulfite buffered at pH 4.5 with 1.0 molar acetate and stored at 80° in sealed, evacuated ampuls.

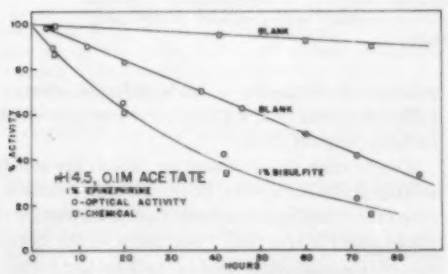

Fig. 11.—Chemical and optical activity of 1.0% epinephrine (0.055 molar) solutions containing bisulfite buffered at pH 4.5 with 0.1 molar acetate and stored at 80° in sealed, evacuated ampuls.

of bisulfite deteriorated solutions which gave these low epinephrine recoveries, a white, crystalline product, m. p. 253–254°, was obtained.

Combustion analysis of the isolated (unacetylated) product indicated the presence of one sulfur atom for each nitrogen atom: C, 42.39%; H, 5.42%; O, 35.36%; N, 5.17%; S, 11.62%. It was insoluble in cold water, 5% hydrochloric acid, 5% sodium bicarbonate, and most organic solvents, and readily soluble in hot water and 5% sodium hydroxide. Iodometric titration of the product and the addition of barium chloride solution failed to show evidence of a sulfite or sulfate salt.

The nonbasic nature of the compound was shown by the following experiment. The product (130 mg.) was dissolved in warm, concentrated hydrochloric acid, and the solution evaporated to a small volume after addition of a small amount of methanol. The white, crystalline precipitate (128 mg.) which separated contained no chlorine, m. p. 259° and gave nearly the same composition as the original product upon analysis: C, 42.13%; H, 5.79%; O, 34.90%; N, 5.47%; S, 10.62%; residue, 2.28%.

The ultraviolet spectrum of the product (Fig. 12) showed an absorbance maximum at 280 mμ in acid solution and at 295 mμ in basic solution, identical in behavior to the parent compound. The infrared spectrum of the compound in potassium bromide disk showed strong absorption bands at 8.3 and 9.7 μ and a band at 6.02 μ.

Upon exhaustive methylation of the product with dimethylsulfate in alkaline solution followed by alkaline permanganate oxidation, 3,4-dimethoxybenzoic (veratric) acid was isolated in 20% yield.

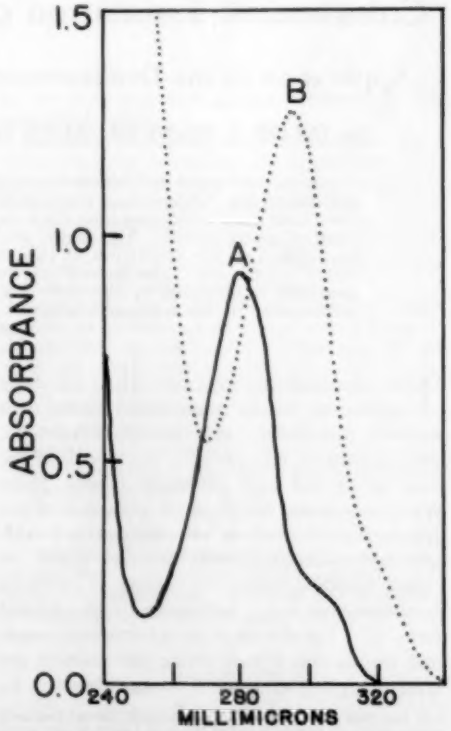

Fig. 12.—Ultraviolet absorption spectra of 1:13,000 solutions of bisulfite product. *A*—acid solution, absorbance maximum 0.91 at 280 mμ; *B*—basic solution, absorbance maximum 1.28 at 295 mμ.

The isolation of veratric acid under these conditions suggested that the sulfur atom was not joined to one of the aromatic carbon atoms.

This evidence supports the belief that the degradation product is formed by an addition of what is equivalent to sulfurous acid to epinephrine. The exact structure is not established at the moment but data indicate some sort of a sulfonate zwitterionic configuration on the side chain. The solubility property of the compound supports this along with the infrared data. The product is inactive both optically and physiologically.

DISCUSSION

At the moment it is difficult to present any rational mechanism which would explain all the observed facts. The individual reactions culminating in the formation of the addition compound have not, as yet, been separated from the kinetics standpoint. The data do point, however, to the existence of a series of steps.

In particular it should be noted that the drug apparently racemizes at a reasonable rate in absence of bisulfite. Yet when bisulfite is added no evidence for racemization is found since the total loss in optical activity can be accounted for by formation of the addition product. If this line of reasoning is correct, it would seem that we have a system in which several competing reactions exist.

Further studies are in progress to obtain additional data concerning the chemical kinetics of this previously unsuspected reaction.

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Colorimetric Estimation of Some Cholinergic Esters*

Application to the Demonstration of Acetylcholinesterase Activity

By JACOB S. HANKER, ALAN GELBERG, and BENJAMIN WITTEN

A procedure for the estimation of acetylcholine, Mecholyl, and succinylcholine by the use of the Schönemann reaction is described. Using this procedure acetylcholinesterase activity and inhibition thereof may be demonstrated by estimation of residual acetylcholine. The theory and scope of the Schönemann reaction in its application to the estimation of choline-type acetates, succinylcholine, and other esters is discussed. The unusual reactivity of the choline-type acetates to hydrogen peroxide, as indicated by a positive Schönemann reaction, is attributed to a unique stabilization of the activated complex by the quaternary nitrogen and perhydroxyl groupings.

THE SCHONEMANN REACTION (1–3) has been applied to the detection and estimation of organic phosphoro- and phosphonohalidates, pyrophosphates, and carbonyl compounds of the acid halide and acid anhydride types. These substances react readily with perhydroxyl ion (peroxide anion) to form unisolable perhydroxylated intermediates, possibly peracids, which are strong oxidizing agents (4) capable of oxidizing benzidine-type redox indicators to the colored form (5). The classes of phosphorus compounds and carbon compounds giving the reaction are good phosphorylating or acylating agents, i. e., substances displaying a high order of electrophilic character of the phosphoryl phosphorus or carbonyl carbon atom.

Esters, such as ethyl acetate, which are much weaker acylating agents, do not give the reaction. However, ethyl acetoacetate had been reported (6) to give the reaction. Inasmuch as the initial step of this reaction involves attack by the nucleophilic perhydroxyl ion, it was felt that esters in which the carbonyl carbon of the carboethoxy group is particularly electrophilic as compared with the corresponding carbonyl carbon of ethyl acetate might be expected to give the Schönemann reaction.

The high electrophilicity of the carbonyl carbon of acetylcholine has been reported (7). This may be due to the contribution of one canonical structure (I) to the resonance hybrid in a cyclic conformation (8).

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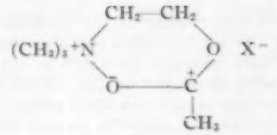
The authors wish to express their appreciation to Drs. Francis M. Miller, George M. Steinberg, and Kenneth B. Wiberg for helpful discussions in the preparation of this manuscript.



I

It was expected, therefore, that choline-type acetates should give the Schönemann reaction.[1] In our investigation, acetylcholine, Mecholyl, and succinylcholine gave strongly positive Schönemann tests. β-Dimethylaminoethyl acetate, in which the carbonyl carbon is much less electrophilic toward hydroxyl ion than in the choline-type acetates (*cf.* relative rates of alkaline hydrolysis (8)) did not give the reaction. Carbachol and bethanechol, choline-type carbamates in which the carbonyl carbon is also much less electrophilic than in the choline-type acetates, did not give the reaction.

The colorimetric method may be used for the quantitative estimation of acetylcholine,[2] Mecholyl, and succinylcholine. By using the reaction for the estimation of residual acetylcholine, acetylcholinesterase activity (Fig. 1) and inhibition thereof[3] may be demonstrated.

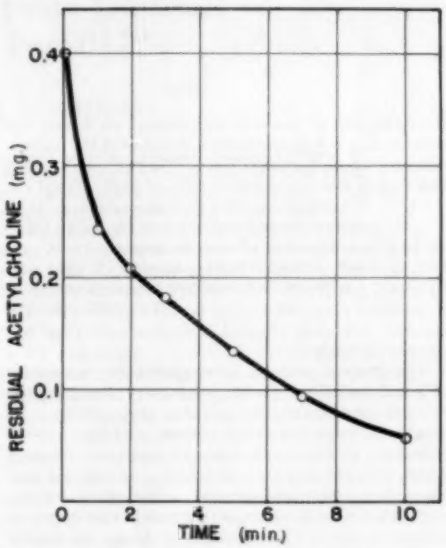


Fig. 1.—Typical plot of residual acetylcholine *versus* time.

EXPERIMENTAL

Estimation of Acetylcholine, Mecholyl, and Succinylcholine

Reagents.—*o*-Dianisidine, 0.1% w/v in acetone, hydrogen peroxide, 3% w/v (unstabilized), and sodium phosphate, tribasic, 0.05 *M*.

Procedure.—A standard curve is prepared by using known concentrations of the ester in 1:1 aqueous acetone. To 1 ml. of the sample solution are added 3 ml. of *o*-dianisidine reagent, 1 ml. hydrogen peroxide solution, and 1 ml. sodium phosphate solution. The color is allowed to develop for four minutes after the addition of the 0.05 *M* sodium phosphate solution and measured with a Beckman Model C Colorimeter using a No. 44 filter.

Demonstration of Acetylcholinesterase Activity

Preparation of Stock Enzyme Solution.—A gelatin-potassium chloride solution is prepared by dissolving 0.2 Gm. gelatin and 4.48 Gm. potassium chloride in 100 ml. water; the solution is boiled thirty minutes (9). Purified bovine erythrocyte acetylcholinesterase,[4] 20,000 units, is dissolved in 10 ml. of the gelatin-KCl solution. This solution is kept refrigerated.

Reagents.—Acetylcholinesterase solution. To 1 ml. of the stock enzyme solution is added 80 ml. of the gelatin-KCl solution; the resulting solution is then diluted to 200 ml. with sterile water.

Acetylcholine chloride, 0.1% w/v.

Sörensen phosphate buffer solution, pH 7.2 (10).

o-Dianisidine, hydrogen peroxide, and tribasic sodium phosphate solutions as above.

Procedure.—To 5 ml. of the phosphate buffer solution in an Erlenmeyer flask is added 10 ml. of acetylcholine chloride solution. Upon the addition of 10 ml. of the diluted enzyme solution, the stop watch is started. At one, two, three, five, seven, and ten-minute intervals, a 1-ml. aliquot of the enzyme-substrate solution is added to a Beckman cylindrical tube containing 3 ml. *o*-dianisidine solution. The procedure given above for the estimation of acetylcholine is then applied.

The residual acetylcholine concentration is read from a calibration curve prepared by applying this procedure to solutions containing 0.04–0.4 mg. acetylcholine chloride per ml. and the Sörensen buffer, but no acetylcholinesterase. A typical plot of residual acetylcholine concentration *versus* time is shown as Fig. 1.

DISCUSSION

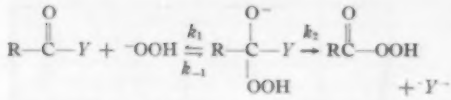
Electrophilicity of the carbonyl carbon is not the only, or even the most important, factor in determining whether an ester will give the Schönemann reaction. Wiberg (11) has suggested that if the reaction of perhydroxyl ion with an ester involves a transition state and the reaction be represented by the equation:

[1] The fact that acetylcholine gives the Schönemann reaction has been independently reported by Aksnes, G., and Sandberg, K., *Acta Chem. Scand.*, **11**, 876(1957).

[2] Iodide ion gives a positive Schönemann reaction; accordingly, iodides of the cholinergic esters cannot be estimated by this method (*cf.* Schwarz, R., and Heinrich, F., *Z. Anorg. Chem.*, **223**, 387(1935)).

[3] A number of acetylcholinesterase inhibitors when present in sufficient quantity (*ca.* 1 μg.) give a positive Schönemann test. The vanishingly small quantities (ca. 10^{-3} μg.) used in demonstrating enzyme inhibition, however, fail to give a positive test.

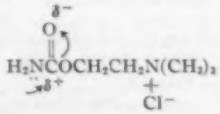
[4] Obtained from Nutritional Biochemicals Corp., Cleveland 28, Ohio.



then the rate of reaction will depend on k_1 and the ratio k_2/k_{-1}. k_1 is primarily a measure of the nucleophilicity of perhydroxyl ion. The ratio k_2/k_{-1} is a function of the relative basicities of perhydroxyl ion + Y^-. If perhydroxyl ion is a stronger base than Y^-, the reaction should proceed and the rate should be largely a function of the nucleophilicity of perhydroxyl ion. Hence, Wiberg found (11) that perhydroxyl ion reacts with phenyl benzoate although it does not react with ethyl acetate, phenol being a stronger acid and ethanol a weaker acid than hydrogen peroxide.

The present authors investigated the reactivity of a number of esters with perhydroxyl ion using the Schönemann reaction as an index of reactivity. As would be expected from Wiberg's study, phenyl benzoate and phenyl acetate gave the reaction whereas ethyl acetate and ethyl benzoate did not. Moreover, ethyl acetoacetate, ethyl chloroacetate, and ethyl trichloroacetate in which the carbonyl carbon atoms of the carboethoxy group are highly electrophilic toward hydroxyl ion actually did not give a positive test under the conditions of the reaction (see Experimental).

However, electrophilicity of the carbonyl carbon is important only when it is of a very low order as in carbachol (II) and bethanechol due to the compensation of the positive charge on the carbonyl carbon by electrons from the amino nitrogen.



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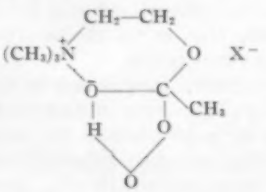
However, electrophilicity of the carbonyl carbon toward hydroxyl ion *per se*, although an excellent guide in this study, is not the reason that the choline-type acetates give the Schönemann reaction. The carbonyl carbon of ethyl trichloroacetate is much more electrophilic toward hydroxyl ion yet it does not give the reaction. Wiberg (12) attributes the reactivity of acetylcholine to the unusual stabilization of the activated complex or transition state by the quaternary nitrogen in which a full positive charge is being developed on the carbonyl oxygen. This stabilization occurs to a much greater extent in the activated complex than the ground state where this type of interaction involves unfavorable separation of charge. Moreover if there were stabilization of the ground state without an even larger effect in the activated state, the reaction would proceed slower instead of faster.

The unusual reactivity to hydrogen peroxide, as indicated by the positive Schönemann reaction, suggests that perhydroxyl ion is more nucleophilic than hydroxyl ion[8] toward the choline-type acetates. It is not inconsistent to propose that the perhydroxyl group in addition to the quaternary nitrogen contributes to the stabilization of the reaction transition state in some unique manner, perhaps as depicted in Structure III.



III

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The Sterol and Triterpene Content of the Labiatae Family*

By HAROLD J. NICHOLAS

The nonsaponifiable fraction of three members of the Labiatae family yielded β-sitosterol. This sterol was accompanied by oleanolic acid and ursolic acid II in *Ocimum basilicum*, and ursolic acid in *Satureia hortensis* and *Hyssopus officinalis*. The diterpene sclareol in *Salvia sclarea* was accompanied by ursolic acid II and oleanolic acid. *Carum carvi* and *Foeniculum vulgare* yielded no acidic triterpenes or identifiable sterols in the nonsaponifiable fraction. The presence of sterols is not precluded, however, in the latter two plants, especially *F. vulgare*. It is believed that more thorough investigation of this plant family may reveal interesting relationships between sterol, diterpene, and triterpene.

ALTHOUGH the weakly acidic fraction of alcoholic extracts of a number of Labiates (mint family) has yielded ursolic acid, oleanolic acid, or ursolic acid II (1, 2), the nonsaponifiable fraction seems to have been little investigated. The present study was undertaken partially to expand some previous observations that sterols, β-sitosterol in particular, sometimes accompany triterpenes in plants (3–6). The survey included one plant already known to contain a diterpene.

EXPERIMENTAL

Materials.—Most of the plants were grown from seed purchased from the Vaughn Seed Company, Chicago. Additional quantities of *Ocimum basilicum* leaves and *Salvia sclarea* were obtained from the Wunderlich-Diez Corporation, New Jersey. For identification purposes authentic ursolic acid and methyl oleanolate were generously supplied by Drs. D. Wenzel and C. Djerassi, respectively. All melting points were determined on a Fisher-Johns melting point apparatus.

Method.—After washing and drying, the whole flowering plant (unless otherwise stated) was finely ground and extracted exhaustively with 95% ethanol. Ethanol extracts were concentrated to a thick gum *in vacuo*. Since previous experience had shown that in *Ocimum basilicum*, *Satureia hortensis*, and *Hyssopus officinalis* only a minor portion of sterol or triterpene occurred as a glycoside (7), acid hydrolysis was not performed and the gummy residue was directly saponified with 10% alcoholic KOH. The cooled mixture was diluted with water, acidified with HCl, and extracted with ethyl ether. The aqueous phase was discarded. The ether extract was then fractionated into strongly acidic material (soluble in 5% $NaHCO_3$), weakly acidic (soluble in or forming an insoluble potassium salt with 5% KOH), and nonsaponifiable material. The nonsaponifiable fraction from the ether was dried *in vacuo*, dissolved in benzene, and poured onto a benzene-washed alumina column (Fisher alumina, 80–200 mesh). A ratio of one part of nonsaponifiables to at least five of alumina was used. The column was then washed with benzene until little color was eluted. Washing the column with ethanol in benzene 2% v/v gave a fraction containing the neutral sterol. This will subsequently be referred to as the "2% ethanol in benzene fraction." The 5% KOH extract of the original ether solution was filtered to remove the partially insoluble potassium salt of oleanolic acid and the very insoluble potassium salt of ursolic acid. The filtrate from this step was acidified to recover ursolic acid II, which does not form an insoluble potassium salt. Most of the microanalyses were performed by Dr. G. Weiler and Dr. F. B. Straus, Microanalytical Laboratory, Oxford, England.

Isolation of β-Sitosterol, Oleanolic Acid, and Ursolic Acid II from *Ocimum basilicum*

Ocimum basilicum (7 Kg.) yielded 71 Gm. of nonsaponifiable material. Chromatography gave a 2% ethanol in benzene fraction consisting of 14.6 Gm. of deep yellow, crystalline solid. After 3 crystallizations from acetone-methanol this gave 4.6 Gm. of white, crystalline solid, m.p. 134–136°. An additional 6.0 Gm., m. p. 138–140°, was obtained from the filtrate by further crystallization.

β-Sitosterol Acetate.—Acetylation of the above 4.6 Gm. with acetic anhydride and anhydrous pyridine (reflux) and crystallization of the product to constant m. p. from acetone and acetone-methanol gave scales, m. p. 126–127°; undepressed on admixture with authentic β-sitosterol acetate. $[\alpha]_D^{20}$ −40° ($CHCl_3$).

β-Sitosterol Benzoate.—The crude free compound (above, 1.3 Gm.) was refluxed with benzoyl chloride and anhydrous pyridine giving white scales on repeated recrystallization from acetone-methanol, m. p. 146–148°; undepressed on admixture with authentic β-sitosterol benzoate. $[\alpha]_D^{20}$ − 14° ($CHCl_3$).

Free β-Sitosterol.—The above acetate (m. p. 123–126°; 2.7 Gm.) was saponified, giving white needles, m. p. 139–140° on repeated recrystallization from acetone-methanol; undepressed on admixture with authentic free β-sitosterol. $[\alpha]_D^{19}$ − 23° ($CHCl_3$).

Isolation of Oleanolic Acid.—The KOH-soluble fraction was acidified yielding 77 Gm. of gummy solid. After drying, this was triturated several times with petroleum ether (b. p. 30–60°); the supernatant was decanted and discarded. Repeated recrystallization of the insoluble residue from methanol eventually gave 10.4 Gm. of white needles, m. p. 302–306°, undepressed on admixture with oleanolic acid. $[\alpha]_D^{20}$ + 78° ($CHCl_3$).

* Received March 24, 1958, from the University of Kansas Medical Center, Department of Obstetrics and Gynecology, Kansas City.

Oleanolic Acid Acetate.—This was prepared by refluxing the free compound with acetic anhydride and pyridine. Needles were subsequently obtained from aqueous ethanol, m. p. 266–268°. $[\alpha]_D^{20}$ + 76° ($CHCl_3$).

Acetyl Methyl Oleanolate.—A methyl ester was prepared by the method of Sando (8). The crude mixture was acetylated with acetic anhydride and anhydrous pyridine and recrystallized to constant m.p. from aqueous methanol and aqueous methanol-acetone giving needles, m.p. 221–223°, undepressed on admixture with authentic material. $[\alpha]_D^{18}$ + 65° ($CHCl_3$).

No crystalline solid of constant m. p. could be isolated from the mother liquor of the original crude mixture or after acetylation, benzoylation, or other treatment. An additional triterpenoid beside oleanolic acid was indicated in the mixture, however.

Isolation of Ursolic Acid II.—An additional 4.5 Kg. of powdered leaves (only) gave a KOH-soluble extract which on acidfication, washing, and drying of the precipitate yielded 55 Gm. of greenish-black gum. This was boiled 3 times with 600 ml. of ligroin (b. p. 90–120°) and the partly cooled mixture was filtered through filter paper. Examination of the combined filtrates gave no indication of oleanolic acid. The ligroin-insoluble material (14.1 Gm.) was refluxed one hour with acetic anhydride and pyridine and the cooled mixture worked up to give 13.8 Gm. of blackish solid. This was dissolved in benzene and poured onto a 200-Gm. benzene-washed alumina column. The latter was washed with benzene until the eluate was almost colorless and then with 2% benzene (12 liters) and 10% ethanol in benzene (12 liters). The ethanol-benzene eluates yielded 7.8 Gm. of greenish solid (a mixture of acetylated and unacetylated material) which was now saponified with 10% alcoholic KOH. The cooled mixture was acidified with HCl and extracted with a large volume of ethyl ether. This was washed with water and distilled. The residue after repeated and slow crystallization from aqueous ethanol gave 3.2 Gm. of semicrystalline material, m.p. 220–250°.

Ursolic Acid II Acetate.—A portion of the above crude mixture was refluxed with acetic anhydride and anhydrous pyridine. Work-up and two crystallizations from aqueous methanol gave white crystals, m. p. 170–175°. Part of this was saponified to obtain the free compound (below). The remainder was crystallized to constant m. p. from absolute ethanol. Needles, m. p. 283–286°; $[\alpha]_D^{20}$ + 60° ($CHCl_3$).

Anal.—Calcd. for $C_{32}H_{50}O_4$: C, 77.06; H, 10.11. Found: C, 77.14, H, 10.28.

Free Ursolic Acid II.—Alkaline saponification and repeated crystallization from aqueous ethanol gave white needles, m.p. 283–285°; $[\alpha]_D^{18}$ + 70° (CH_3OH).

Anal.—Calcd. for $C_{30}H_{48}O_3$: C, 78.88; H, 10.61. Found: C, 78.90; H, 10.40.

Ursolic Acid II Methyl Ester.—Prepared according to the method of Sando (8). Several crystallizations from aqueous ethanol gave m. p. 168–170°; $[\alpha]_D^{18}$ + 60° (CH_3OH).

Brieskorn and Eberhardt (2) give the following constants for ursolic acid II and its derivatives: free compound, m. p. 284–285°; $[\alpha]_D^{21}$ + 72.4°, (CH_3OH). Acetate, m. p. 286°; $[\alpha]_D^{21}$ + 62.5° Methyl ester, m. p. 169–170°; $[\alpha]_D$ + 62.7°.

Isolation of β-Sitosterol and Ursolic Acid from *Satureia hortensis*

The dried plant (1,117 Gm.) yielded a nonsaponifiable fraction weighing 8.5 Gm. This on chromatography gave a 2% ethanol in benzene fraction consisting of 2.0 Gm. of red wax. Repeated crystallization from acetone and acetone-methanol produced white needles, m. p. 139–141°, undepressed on admixture with authentic β-sitosterol. $[\alpha]_D^{20}$ −26° ($CHCl_3$). Acetylation in the manner described above gave white scales, m. p. 126–127°, $[\alpha]_D^{18}$ −40° ($CHCl_3$), and benzoylation gave white scales, m. p. 144–145°, after several crystallizations from acetone-methanol. Neither of these derivatives had its m. p. depressed on admixture with authentic material.

The KOH-soluble extract was filtered, giving 0.9 Gm. of white, insoluble solid (acidification of the filtrate produced no solid). This was washed with water dried, and dissolved in hot absolute ethanol. The cooled mixture was acified with HCl, and the precipitate crystallized several times from absolute ethanol to give white needles, m. p. 283–285°; $[\alpha]_D^{21}$ + 66° ($CHCl_3$).

Ursolic Acid Acetate.—Prepared according to directions in (3), needles m. p. 285–286°.

Ursolic Acid Methyl Ester.—Prepared according to the procedure of (8); needles, m. p. 165–167°. Brieskorn, *et al.* (1), have previously reported the presence of ursolic acid in *Satureia hortensis*.

Isolation of β-Sitosterol and Ursolic Acid from *Hyssopus officinalis*

This plant (3,600 Gm.) gave 13.2 Gm. of nonsaponifiable fraction which yielded 4.9 Gm. of 2% ethanol in benzene fraction as a deep red wax. Several crystallizations from acetone and acetone-methanol gave white needles, m. p. 139–140°; $[\alpha]_D^{18}$ − 37° ($CHCl_3$). From this free compound an acetate, m. p. 125–128°, and a benzoate, m. p. 145–147°, were obtained. The m.p.'s of these compounds were not depressed by admixture with authentic β-sitosterol, β-sitosterol acetate, or β-sitosterol benzoate, respectively.

The KOH-soluble material on filtration gave a precipitate (7.0 Gm.) which when treated like the corresponding solid from *Satureia* yielded white needles, m. p. 285–287°, $[\alpha]_D^{20}$ + 68° ($CHCl_3$), undepressed on admixture with authentic ursolic acid

Ursolic Acid Acetate.—Prepared as indicated for *Satureia*. Needles, m. p. 280–290°.

Ursolic Acid Methyl Ester.—Prepared as indicated for *Satureia*. Needles, m. p. 164–167°. Brieskorn, *et al.* (1), have previously reported the presence of ursolic acid in *Hyssopus officinalis*.

Isolation of Sclareol, Ursolic Acid II, and Oleanolic Acid from *Salvia sclarea*

The plant material as received contained much evidence of flower parts, despite being labeled leaves. This may account for the presence of oleanolic acid. In a recent examination of a much larger quantity of *Salvia sclarea* leaves only, no oleanolic acid was indicated. The ground plant (2,270 Gm.) yielded 34.0 Gm. of nonsaponifiable fraction. From this 9.5 Gm. of 2% ethanol in benzene fraction was isolated as a greenish wax. A portion of this (1.28 Gm.) was sublimed in a cold finger apparatus at 10 μ Hg pressure. Two fractions of 0.47 and 0.43 Gm., respec-

tively, were obtained over two 4-hour periods at 90° pot temperature. A nondistilled residue of 0.32 Gm. is being investigated. The combined sublimates were crystallized several times from aqueous methanol and ligroin yielding white needles, m. p. 104–105°; $[\alpha]_D^{18} - 8.7°$ ($CHCl_3$).

Anal.—Calcd. for $C_{28}H_{28}O_2$: C, 77.86; H, 11.76. Found: C, 77.65; H, 11.80. Simonsen and Barton give m. p. 105–106°, $[\alpha]_D - 3.3°$ ($CHCl_3$) for sclareol (9). The product gives the color reactions described by Volmar and Jermstad (10).

The KOH extract was filtered, giving 0.5 Gm. of potassium salt. After washing and drying this was subsequently treated like the potassium salt from *Satureia*, yielding white needles, m. p. 304–306°, undepressed on admixture with authentic oleanolic acid. $[\alpha]_D^{18} + 75°$ ($CHCl_3$).

Oleanolic Acid Acetate.—Prepared as described for that from *Ocimum basilicum*. Needles, m. p. 265–268°. $[\alpha]_D^{20} + 76°$ ($CHCl_3$).

Oleanolic Acid Methyl Ester Acetate.—Prepared as described for the same compound from *O. basilicum*, m. p. 223–224°; undepressed on admixture with authentic material. $[\alpha]_D^{20} + 67°$ ($CHCl_3$).

Ursolic Acid II.—Acidification (with HCl) of the filtered KOH extract gave a voluminous precipitate which was filtered, washed, and dried. From the 3.5 Gm. of greenish solid so obtained, 1.2 Gm. of ursolic acid II was eventually procured by modifications of the procedure described for isolation of ursolic acid II from *Ocimum basilicum:* needles, m. p. 285–286°, $[\alpha]_D^{20} + 72°$ (CH_3OH).

Anal.—Calcd. for $C_{30}H_{48}O_3$: C, 78.88; H, 10.61. Found: C, 78.60; H, 10.50.

Ursolic Acid II Acetate.—Obtained as previously described for similar material from *Ocimum basilicum:* needles, m. p. 284–286°, $[\alpha]_D^{18} + 61°$ (CH_3OH).

Ursolic Acid II Methyl Ester.—M. p. 165–168° when prepared as previously described.

Examination of *Carum carvi*

The ground plant (400 Gm.) yielded 3.0 Gm. of nonsaponifiable fraction as a brown wax. From this a 2% ethanol in benzene fraction of 1.8 Gm. was obtained. This orange wax on repeated crystallization from acetone-methanol mixtures gave white microcrystals, m. p. 70–72°, $[\alpha]_D^{23} - 50°$ ($CHCl_3$). Since it seemed quite unlikely that this was even grossly impure sitosteroi, it was not further investigated.

The KOH-soluble fraction on acidification gave 0.5 Gm. of gummy solid. It was completely and readily soluble in warm acetone, and no evidence of any acidic triterpenes was indicated. However, when spotted from solvent on paper and then sprayed with 10% $SbCl_3$ in $CHCl_3$, a black color was obtained. In our hands β-sitosterol, stigmasterol, and sclareol also give this color reaction, and it is possible that a sterol, diterpene, or fragment thereof was present.

Examination of *Foeniculum vulgare*

The ground plant (3,000 Gm.) yielded 7.2 Gm. of nonsaponifiable fraction as a yellow wax with a pleasant odor. From this a 2% ethanol in benzene fraction of 6.0 Gm. was obtained as a yellow solid. Repeated crystallization from acetone and acetone-methanol gave white crystals melting unsharply at 109–125°. Acetylation of this solid produced transparent scales on several crystallizations from acetone-methanol, m. p. 124–125°; $[\alpha]_D^{18} - 45°$ ($CHCl_3$). No material of constant m. p. was obtained by saponification of this acetate and repeated crystallization from acetone-methanol. With the Liebermann-Burchard reagent (chloroform, acetic anhydride underlayed with concd. H_2SO_4) a red interface developed within twenty minutes, associated with a transient yellow color in the chloroform layer. It seems highly probable that this substance is a sterol, but positive identification was not made.

The KOH-soluble material contained no insoluble salt and on acidification with HCl gave 3.8 Gm. of brown wax with a pleasant odor. No evidence of acidic triterpenes was given. A spot on paper gave a black color with $SbCl_3$ in $CHCl_3$.

RESULTS AND DISCUSSION

It is, of course, not possible to develop any biogenetic relationship between sterol, diterpene, and triterpene and the particular species of plants containing them from the few plants examined. However, it is interesting that although sterol and triterpene, respectively, and diterpene and triterpene were found together, no diterpene and sterol accompanied each other in an individual plant. A survey of the literature on the Labiatae gives no further information to expand these data. In another plant family, the occurrence of a diterpene (marrubiin) and a triterpene (ursolic acid) in *Marrubium vulgare* L. follow this sequence (1, 11).

The constitution of ursolic acid II is not known, but it appears to be a close structural isomer of ursolic acid. Ursolic acid II was obtained pure only with considerable difficulty in the presence of high concentrations of oleanolic acid.

Sclareol is best obtained from *Salvia sclarea* by extraction of the dried plant with ligroin, from which sclareol is subsequently isolated by fractional distillation. The saponification instituted in our experimental procedure was designed for isolation of sterol, not diterpenes. It is rather surprising that such a good yield of the tertiary alcohol was obtained after saponification.

Further investigation of the Labiatae family for sterol, diterpene, and triterpenes may prove promising for the development of biogenetic relationships between these terpenoid substances and the various species containing them within this family.

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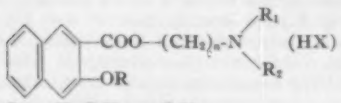
Dialkylaminoalkyl Esters and Amides Derived from 3-Hydroxy- and 3-Alkoxy-2-naphthoic Acids*

By GEORGE M. SIEGER,† WILLIAM M. ZIEGLER,† DAVID X. KLEIN, and HERMAN SOKOL

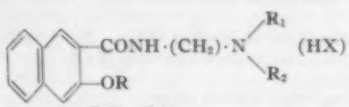
A number of dialkylaminoalkyl esters and amides derived from 3-hydroxy- and 3-alkoxy-2-naphthoic acids have been synthesized and characterized as hydrochlorides, organic acid salts, and quaternary ammonium derivatives. The results of preliminary physiological and bacteriological screening tests on these compounds have been reported.

IN CARRYING out a literature survey on local anesthetics it was noted that Fisk and Underhill (1) and Bjerregaard and Houston (2) reported that α- and β-naphthoic acids possess anesthetic properties. Because of the interest of these laboratories in derivatives of 3-hydroxy-2-naphthoic acid (beta-oxynaphthoic acid), these references prompted the preparation of procaine (3) and Nupercaine (4, 5) analogs for general physiological studies with special emphasis on potential local anesthetic properties. Accordingly, it was decided to synthesize several series of dialkylaminoalkyl esters and amides derived from 3-hydroxy- and 3-alkoxy-2-naphthoic acids (and their inorganic and organic acid salts) represented by general formulas A and B. It followed that quaternary ammonium salts of the basic esters and amides, described by formulas C and D, should also be prepared for general physiological and bacteriological testing with a particular interest in possible antispasmodic and anticholinergic properties.



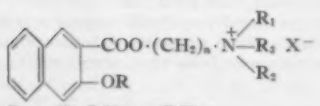
R = H, C_2H_5, or C_4H_9
R_1, R_2 = C_2H_5, C_4H_9, n = 2 or 3
HX = Acid salts, e.g., HCl, tartaric acid, etc.

(A)



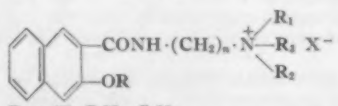
R = H, C_2H_5, C_4H_9
R_1 = H, C_2H_5
R_2 = C_2H_5, $CH(CH_3)_2$, n = 2 or 3
HX = Acid salts, e.g., HCl, tartaric acid, etc.

(B)



R = H, C_2H_5, or C_4H_9
R_1, R_2 = C_2H_5, n = 2 or 3
R_3 = CH_3, $CH_2C_6H_5$
X^- = Br^-, Cl^-

(C)



R = H, C_2H_5, C_4H_9
R_1, R_2 = C_2H_5, n = 2 or 3
R_3 = CH_3, C_2H_5, $CH_2C_6H_5$
X^- = I^-, Br^-, or Cl^-

(D)

EXPERIMENTAL DESIGN

The synthesis of the esters (series A) was accomplished by reaction of the respective acid chlorides with the desired amino alcohol. Treating 3-hydroxy-2-naphthoic acid (I) with thionyl chloride in petroleum ether forms the corresponding acid chloride (IV), which is then treated with 2-diethylaminoethanol hydrochloride in chloroform according to the esterification method of Cope (6) to give 2-diethylaminoethyl 3-hydroxy-2-naphthoate hydrochloride (VI). Compound VI can also be obtained directly from the acid I by treatment with the free base of 2-diethylaminoethyl chloride in isopropyl alcohol according to an esterification method reported by Burtner and Cusic (7). A modification of the first procedure given, in which the free base of the amino alcohol was used in place of the hydrochloride, was described by Robinson (8), who prepared several aminoalkyl esters of naphthoic and hydroxynaphthoic acids for the pharmacological studies of Fisk and Underhill (1). Robinson (8) prepared the 2-diethylaminoethyl and 3-diethylaminopropyl-3-hydroxy-2-naphthoates (hydrochlorides), reporting melting points of 165–185° and 178–180°, respectively, as compared to corresponding values of 192.2–193.7° and 184–184.6° (compounds VII and VI in Table I) found in this laboratory; and he

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prepared the 3-diethylaminopropyl and 2-diethylaminoethyl esters of 3-ethoxy-2-naphthoic acid (hydrochlorides), giving melting points of 139–140° and 145–146°, respectively, as compared to 139.5–141.5° and 144.5–146.5° (compounds XVI and XV in Table I) found in this laboratory. The results obtained by Fisk and Underhill (1) indicated that the 3-diethylaminopropyl 3-ethoxy-2-naphthoate was superior to the other compounds tested, being the least irritating, having fair stability in solution and good anesthetic power but that the corresponding 3-hydroxy compound was the most irritating and that its aqueous solution darkened readily. These findings were at variance with our results. Compound VII was found to have the best therapeutic index of all the esters tested; it was relatively nonirritating and had fair stability. It is believed that these differences in the results can be readily explained by the fact that our product was pure whereas Robinson's material, as the melting point indicates, contained impurities.

The synthesis of the amides (series B) was accomplished by converting the acids in a suitable solvent to the acid chlorides, which in turn were treated with various aminoalkylamines. For example, the 3-hydroxy-2-naphthoyl chloride (IV) on treatment with N,N-diethylethylene diamine (2-diethylaminoethylamine) in benzene solution gave N - (2 - diethylaminoethyl) - 3 - hydroxy - 2 - naphthoamide hydrochloride (XIX). The amides like the esters were found to be good anesthetics and had the advantage of being more stable in aqueous solutions, the esters being more susceptible to hydrolysis.

The quaternary ammonium salts (series C and D) were prepared by reaction of various alkyl and aralkyl halides with the free bases of the basic esters and amides (series A and B).

PHARMACOLOGY

The dialkylaminoalkyl esters and amides derived from 3-hydroxy- and 3-alkoxy-2-naphthoic acids were found to possess a high order of anesthetic activity, superior to that of procaine, as can be observed in Tables V and VI (26). The 3- and 2-diethylaminopropyl and the 3-dibutylaminopropyl esters of 3-hydroxy-2-naphthoic acid (VII, X, XII) were found to be the most active and were found to be less toxic than procaine in the subcutaneous and intraperitoneal tests.

In general, it was found that the corresponding esters in the 3-ethoxy series were approximately of equal potency but were less stable and more irritating. These anesthetic tests were carried out on the cornea of the rabbit (11) and the sciatic nerve of the guinea pig, and in addition a retrobulbar block test was run on the amides (12). The amides (series B) were in general found to possess the same order of activity as the corresponding esters (series A) but were found to be more toxic. It was noticed that the toxicity of a particular amide increases with the size of the 3-alkoxy group going from 3-hydroxy to 3-ethoxy to 3-butoxy substituents. The amides are not as readily hydrolyzed as the esters and are, therefore, more stable in aqueous solutions.

In the amide series N-(2-diethylaminoethyl)-3-hydroxy-2-naphthoamide hydrochloride (XIX) and N - (3 - diethylaminopropyl) - 3 - hydroxy - 2-naphthoamide hydrochloride (XX), or its *d*-tartaric acid salt (XXI), appeared to be potent, relatively harmless anesthetics affording much greater anesthesia than that of procaine as indicated in Table VI. Compound XXVII, a Nupercaine analog, namely, N - (2 - diethylaminoethyl) - 3 - butoxy - 2 - naphthoamide, a powerful anesthetic, was found to be more toxic than procaine but less harmful than Nupercaine. In preliminary clinical tests (13–16), selected membe:s[1] of the basic ester and amide series (A and B) have given results that are sufficiently favorable to warrant further investigation of these compounds.

Compounds of series A, B, C, and D were screened for antispasmodic properties; those which had anticholinergic activity that compared favorably with that of Trasentine, Panparnit, and Syntropan, in preventing spasm produced by acetylcholine in the isolated ileum of the guinea pig, according to techniques described in the literature (17–19), were N-(3-diethylaminopropyl) 3-butoxy-2-naphthoamide (XXVIII), N-(3-dimethylaminopropyl) 3-butoxy-2-naphthoamide (XXX), N-(2-diethylaminoethyl) 3-hydroxy-2-naphthoamide benzyl chloride (XXXIX), N-(3-diethylaminopropyl)-3-butoxy-2-naphthoamide benzyl chloride (XLV), and 3-diethylaminopropyl 3-butoxy-2-naphthoate benzyl chloride (XXXVI). However, Banthine and atropine sulfate demonstrated activity far superior to any of these compounds under similar conditions.

None of the compounds tested as blocking agents in preventing fatal bronchiospasm in guinea pigs exposed to histamine aerosol according to the method of Loew, Kaiser, and Moore (19) demonstrated sufficient antihistaminic activity in comparison with Pyribenzamine to warrant further study.

BACTERIOLOGICAL RESULTS

A study was made to compare the relative antimicrobial properties of 3-diethylamino-propyl-3-hydroxy-2-naphthoate hydrochloride (VII) and 2-diethylaminopropyl-3-hydroxy-2-naphthoate hydrochloride (X) with several well-known local anesthetics because it was postulated that members of series A and B with a free phenolic hydroxyl group might conceivably have an antibacterial effect in addition to producing anesthesia. It was found that of six local anesthetics tested that Xylocaine was the most effective agent inhibiting all six test bacteria[2] at a dilution of 1:3,200, followed by compound VII (1:1,600), Pontacaine (1:1,600), compound X (1:800), and Intracaine (1:200), all inhibiting five of the six test organisms at the dilution indicated. Procaine only inhibited two of the six at a dilution of 1:100.

Using a modified phenol coefficient procedure,[3] it

[1] These compounds have been submitted for clinical testing as local anesthetics under the name Bonacaines, which is derived from Bona, an abbreviation for beta-oxynaphthoic acid, or 3-hydroxy-2-naphthoic acid (this acid is available at the Heyden Newport Chemical Corporation). Bonacaine A is compound XIX; Bonacaine B is compound X; Bonacaine C is compound XXVII; Bonacaine G is compound VII, and Bonacaine AG is compound XX. In the Graubard, *et al.* (14), article on Bonacaine A this compound was incorrectly called alpha diethylaminopropyl 2-hydroxy-3-naphthoate; it should have read N-(2-diethylaminoethyl) 3-hydroxy-2-naphthoamide.

[2] The test organisms were *K. pneumoniae*, *E. coli*, *B. subtilis*, *Ps. fluorescens*, *Prot. vulgaris*, and *S. aureus*. The agar-dilution method was used to compare the antibacterial properties.

[3] Based largely on procedure given by Ruehle and Brewer, U. S. D. A. Circular No. 198.

TABLE I.—DIALKYLAMINOALKYL ESTERS OF 3-HYDROXY- AND 3-ALKOXY-2-NAPHTHOIC ACIDS (SERIES A)

No.	Acid	Amino Alcohol	Salt	M. p., ° C., Corrected	Formula	Nitrogen Calcd.	Nitrogen Found	Chlorine Calcd.	Chlorine Found	Yield, %	Solvent Used For Crystallization
VI	3-Hydroxy-2-naphthoic	2-Diethylaminoethanol	HCl	184–184.6	$C_{17}H_{22}ClNO_3$	4.3	4.3	11.0	11.2	65	$CHCl_3$ or Ethanol
VII	3-Hydroxy-2-naphthoic	3-Diethylaminopropanol	HCl	192.2–193.7	$C_{18}H_{24}ClNO_3$	4.13	4.05	10.5	10.48	43	2-Propanol
VIII	3-Hydroxy-2-naphthoic	3-Diethylaminopropanol	*d*-Bitartrate[b]	.....	$C_{22}H_{29}NO_9$	3.72	3.65	..	..	Quantitative	H_2O-$CHCl_3$
IX	3-Hydroxy-2-naphthoic	3-Diethylaminopropanol	*d*-Tartrate[b]	.....	$C_{40}H_{52}N_2O_{12}$[c]	3.66	3.63	..	..	Quantitative	H_2O-$CHCl_3$
X	3-Hydroxy-2-naphthoic	2-Diethylaminopropanol	HCl	159.7–161.2	$C_{18}H_{24}ClNO_3$	4.13	4.0	10.5	10.28	54	2-Propanol
XI	3-Hydroxy-2-naphthoic	2-Dibutylaminoethanol	HCl	155–156	$C_{21}H_{30}ClNO_3$	3.69	3.71	9.3	9.4	45	2-Propanol
XII	3-Hydroxy-2-naphthoic	3-Dibutylaminopropanol	HCl	152–154	$C_{22}H_{32}ClNO_3$	3.56	3.59	9.0	9.2	45	2-Propanol
XIII	3-Hydroxy-2-naphthoic	2-Dibutylaminopropanol	HCl	154–155	$C_{22}H_{32}ClNO_3$	3.56	3.76	9.0	9.2	53	2-Propanol
XIV	3-Hydroxy-2-naphthoic	3-Diethylamino-2,2-dimethylpropanol[a]	HCl	173–173.5	$C_{20}H_{28}ClNO_3$	3.8	3.7	9.7	9.8	55	2-Propanol
XV	3-Ethoxy-2-naphthoic	2-Diethylaminoethanol	HCl	144.5–146.5	$C_{19}H_{26}ClNO_3$[c]	..	..	..	..	72	$CHCl_3$-Ethyl acetate
XVI	3-Ethoxy-2-naphthoic	3-Diethylaminopropanol	HCl	139.5–141.5	$C_{20}H_{28}ClNO_3$[c]	..	..	..	..	90	$CHCl_3$-Ethyl acetate
XVII	3-Ethoxy-2-naphthoic	2-Dibutylaminoethanol	HCl	140.5–142.5	$C_{23}H_{34}ClNO_3$	..	..	..	..	75	$CHCl_3$-Ethyl acetate
XVIII	3-Butoxy-2-naphthoic	3-Diethylaminopropanol	HCl	106.5–109.1	$C_{22}H_{32}ClNO_3$	3.56	3.62	9.0	9.4	59.2	Ethyl acetate

[a] This amino alcohol was reported by Mannich (9) and was used to prepare Larocaine.
[b] Other organic acids of this basic ester prepared were the salicylate, bisuccinate, succinate, and bimaleate.
[c] These compounds were prepared by Hill, *et al.* (8), and studied by Fisk and Underhill (1). Fenwick and Gilman (10) determined the acidity constants (KW/KB) of aqueous solutions of the hydrochlorides of compounds XV and XVI at 25°.

TABLE II.—AMINOALKYL AMIDES OF 3-HYDROXY- AND 3-ALKOXY-2-NAPHTHOIC ACIDS (SERIES B)

No.	Acid	Aminoalkylamine	Salt	M. p., ° C., Corrected	Formula	Nitrogen Calcd.	Nitrogen Found	Chlorine Calcd.	Chlorine Found	Yield, %	Solvent Used for Crystallization
XIX	3-Hydroxy-2-naphthoic	2-Diethylaminoethylamine	HCl	156.5–157.6	$C_{17}H_{23}ClN_2O_2$	8.7	8.6	11.0	11.0	69	2-Propanol—Benzene
XX	3-Hydroxy-2-naphthoic	3-Diethylaminopropylamine	HCl[a]	Resinous	$C_{18}H_{25}ClN_2O_2$	8.3	8.1	10.6	10.4	77	.....
XXI	3-Hydroxy-2-naphthoic	3-Diethylaminopropylamine	*d*-Tartrate	156–157	$C_{30}H_{34}N_4O_{10}$	7.47	7.53	..	..	67.3	Ethanol

XXII	3-Hydroxy-2-naphthoic	3-Diethylaminopropylamine	*d*-Bitartrate[a]	Above 70° (decompn.)	$C_{20}H_{30}N_2O_8$	6.22	6.11	..	..	Ethanol
XXIII	3-Hydroxy-2-naphthoic	3-Dimethylaminopropylamine[b]	Free base	153–155	$C_{18}H_{26}N_2O_2$	10.3	10.1	..	59.2	Aqueous 2-propanol
XXIV	3-Hydroxy-2-naphthoic	3-Isopropylaminopropylamine[b]	HCl	205.7–207.1	$C_{17}H_{28}ClN_3O_2$	8.7	8.5	11.0	11.2	40	Ethanol-Ether
XXV	3-Ethoxy-2-naphthoic	2-Diethylaminoethylamine	HCl	150–154.5	$C_{18}H_{28}ClN_3O_2$	8.0	7.7	10.1	10.0	47.2	2-Propanol—Ethyl acetate
XXVI	3-Ethoxy-2-naphthoic	3-Diethylaminopropylamine	Free base	59.8–60.9	$C_{20}H_{32}N_2O_2$	8.54	8.44	..	..	78	Benzene-Petroleum ether
XXVII	3-Butoxy-2-naphthoic	2-Diethylaminoethylamine	Free-base	75–77	$C_{20}H_{32}N_2O_2$	8.2	8.1	..	..	72.5	Benzene-2-Propanol
XXVIII	3-Butoxy-2-naphthoic	3-Diethylaminopropylamine	Free base	53–55	$C_{22}H_{36}N_2O_2$	7.87	7.63	..	..	75	HCl-NH_4OH
XXIX	3-Butoxy-2-naphthoic	3-Diethylaminopropylamine	$HCl\text{-}H_2O$[c]	135–136	$C_{22}H_{39}ClN_2O_3$	6.83	6.76	8.66	8.64	65.2	Dilute HCl Solution
XXX	3-Butoxy-2-naphthoic	3-Dimethylaminopropylamine	Free base	81–83	$C_{20}H_{32}N_2O_2$	8.5	8.6	..	..	55	Benzene-Petroleum ether

[a] These salts were of a resinous consistency. [b] These materials were supplied by the American Cyanamid Company. [c] Isolated as a monohydrate. H_2O Calcd., 4.4; H_2O Found, 5.0.

was found that none of the compounds at a 1% concentration killed *S. aureus* with a maximum exposure time of eight minutes. As for antifungal properties, both 3- and 2-diethyl-aminopropyl 3-hydroxy-2-naphthoate hydrochlorides failed to inhibit the growth of *A. niger* at a concentration of 0.04% under the same conditions in a honey broth in which it was found that propyl *p*-hydroxybenzoate was active at a 0.02% level.

As for the quaternary ammonium salts, results of primary antibacterial and antifungal screening tests are recorded in Table VII. It may be observed in comparison with G-11 and G-4 the only two compounds of those tested that exhibited any significant antibacterial activity were 3-diethylaminopropyl 3-hydroxy-2-naphthoate benzyl chloride (XXXIV) and N-(3-dimethylaminopropyl)-3-butoxy-2-naphthoamide benzyl chloride (XLVI), which were also found to be active against *S. aureus* 209-P, *M. phlei* 355, *M. tuberculosis* 607, and *M. smegmatis* 361 in secondary tests both in plain agar and agar containing 5% blood at dilutions of 1:10,000 as compared to G-11, being found active at dilutions of 1:1,000,000–1:10,000,000 in plain agar and 1:10,000 in the presence of blood.

EXPERIMENTAL

Synthesis of the Acids

The preparation of the 3-alkoxy-2-naphthoic acids involved the preparation of ethyl 3-hydroxy-2-naphthoate (I-a), m. p.: 85°, according to the method of Cohen and Dudley (22), which was converted to the sodium salt in alcoholic solution under anhydrous conditions, then alkylated with the respective alkyl halides to form the ethers, and finally the ester group was hydrolyzed to give the desired alkoxy-acid.

Ethyl 3-hydroxy-2-naphthoate, I-2, (22).—Into a 3-liter, 3-necked, round-bottomed flask, fitted with stirrer, thermometer, and condenser, were placed 250 Gm. (1.33 moles) of 3-hydroxy-2-naphthoic acid[4] (I), 1,000 ml. of dry ethanol, and 50 ml. of concentrated sulfuric acid. After refluxing three hours at 83–84°, most of the excess alcohol was distilled and the oily concentrate was poured into 1 liter of cold water. On stirring, the product solidified. The crude product was recrystallized from 500 ml. of hot isopropyl alcohol. Yield, 248 Gm. (86%). The product melted 78.3 to 82.8°. After a second recrystallization it melted 82–83°.

Anal.—Calcd. for $C_{13}H_{12}O_3$: Ester No. 259.6 mg. KOH/Gm. Found: Ester No. 259.7 mg. KOH/Gm.

3-Ethoxy-2-naphthoic Acid, II, (23).—Into a 3-liter, round-bottomed flask equipped with thermometer, reflux condenser, dropping funnel, and stirrer, was placed 1,000 ml. of anhydrous 2B ethanol. Sodium metal (27.6 Gm., 1.2 moles) was added in small slices to the alcohol, while stirring, until all of the sodium was dissolved. Ethyl beta-oxynaphthoate (216 Gm., 1.0 mole) was introduced into the alcoholic solution of sodium ethylate, followed by 5 Gm. of sodium iodide. With continuous stirring

[4] Additional experimental data relating to the studies on local anesthetics conducted at the Research Laboratories of the Heyden Newport Chemical Corporation may be found in references (26) and (27).

TABLE III.—QUATERNARY AMMONIUM SALTS OF DIALKYLAMINOALKYL ESTERS DERIVED FROM 3-HYDROXY- AND 3-ALKOXY-2-NAPTHOIC ACIDS (SERIES C)

No.	Acid	Amino Alcohol	Salt	M. p., ° C.	Formula	Nitrogen Calcd.	Nitrogen Found	Halogen Calcd.	Halogen Found	Yield, %	Solvent Used For Crystallization
XXXI	3-Hydroxy-2-naphthoic	2-Diethylamino-ethanol	CH_3Br	169.1–170.8	$C_{19}H_{24}BrNO_3$	3.66	3.62	20.9	20.8	75	2-Propanol-Ethanol
XXXII	3-Hydroxy-2-naphthoic	2-Diethylamino-ethanol	$C_6H_5CH_2Cl$	150.1–151.1	$C_{24}H_{28}ClNO_3$	3.38	3.27	8.59	8.57	40.3	2-Propanol-Acetone-Ether
XXXIII	3-Hydroxy-2-naphthoic	3-Diethylamino-propanol	CH_3Br	158.2–159.3	$C_{19}H_{26}BrNO_3$	3.54	3.52	20.2	19.9	73.6	2-Propanol
XXXIV	3-Hydroxy-2-naphthoic	3-Diethylamino-propanol	$C_6H_5CH_2Cl$	172.1–173.5	$C_{25}H_{30}ClNO_3$	3.3	2.9	8.3	8.2	51.6	Toluene
XXXV	3-Hydroxy-2-naphthoic	2-Diethylamino-propanol	CH_3Br	168.3–170.8	$C_{19}H_{26}BrNO_3$	3.54	3.47	20.2	20.1	78.1	2-Propanol
XXXVI	3-Butoxy-2-naphthoic	3-Diethylamino-propanol	$C_6H_5CH_2Cl$	Resinous[a]	$C_{29}H_{38}ClNO_3$	2.9	2.9	7.3	6.6	50	.....

[a] This material did not crystallize; it was worked up by dissolving the crude product in $CHCl_3$ solution, filtering the solution and removing the volatiles by distillation *in vacuo*.

TABLE IV.—QUATERNARY AMMONIUM SALTS OF DIALKYLAMINOALKYL AMIDES DERIVED FROM 3-HYDROXY- AND 3-ALKOXY-2-NAPHTHOIC ACIDS (SERIES D)

No.	Acid	Aminoalkylamine	Salt	M. p., °C.	Formula	Nitrogen Calcd.	Nitrogen Found	Halogen Calcd.	Halogen Found	Yield, %	Solvent Used for Crystallization
XXXVII	3-Hydroxy-2-naphthoic	2-Diethylamino-ethylamine	CH_3Br	147–150.3	$C_{18}H_{23}BrN_2O_2$	7.37	7.27	21.0	20.7	72.1	Ethyl acetate
XXXVIII	3-Hydroxy-2-naphthoic	2-Diethylamino-ethylamine	C_2H_5Br	65–70	$C_{19}H_{27}BrN_2O_2$	7.1	6.7	20.3	19.3	49	Toluene-Benzene
XXXIX	3-Hydroxy-2-naphthoic	2-Diethylamino-ethylamine	$C_6H_5CH_2Cl$	64–70	$C_{24}H_{29}ClN_2O_2$	6.78	6.74	8.62	8.77	52	Toluene-Benzene
XL	3-Ethoxy-2-naphthoic	2-Diethylamino-ethylamine	CH_3Br	Resinous[a]	$C_{20}H_{29}BrN_2O_2$	6.8	6.4	19.5	19.3	72.6	.....
XLI	3-Ethoxy-2-naphthoic	3-Diethylamino-propylamine	CH_3Br	122–127	$C_{21}H_{31}BrN_2O_2$	6.62	6.44	18.9	19.4	69	Chloroform
XLII	3-Butoxy-2-naphthoic	2-Diethylamino-propylamine	CH_3I	145.2–146.4	$C_{22}H_{33}IN_2O_2$	5.78	5.62	26.2	25.9	69	Ethanol-Ether
XLIII	3-Butoxy-2-naphthoic	2-Diethylamino-propylamine	$C_6H_5CH_2Cl$	Resinous[a]	$C_{29}H_{37}ClN_2O_2$	5.73	5.47	7.58	7.22	51	.....
XLIV	3-Butoxy-2-naphthoic	3-Diethylamino-propylamine	CH_3Br	101–116[b]	$C_{23}H_{35}BrN_2O_2$	6.22	6.15	17.8	17.9	63.5	$CHCl_3$-Ether
XLV	3-Butoxy-2-naphthoic	3-Diethylamino-propylamine	$C_6H_5CH_2Cl$	Resinous[a]	$C_{29}H_{39}ClN_2O_2$	5.8	5.3	7.4	7.0	62.5	.....
XLVI	3-Butoxy-2-naphthoic	3-Dimethylamino-propylamine	$C_6H_5CH_2Cl$	103–105	$C_{27}H_{35}ClN_2O_2$	6.17	5.82	7.82	7.82	72.2	2-Propanol—Ethyl acetate-Ether

[a] This material did not crystallize; it was isolated from a $CHCl_3$ solution by the method described in Table III, footnote *a*. [b] Hygroscopic.

211 Gm. (1.35 moles) of ethyl iodide was now added through the dropping funnel. The resulting reaction mixture was refluxed for about sixteen hours (temperature, *ca.* 80–83°).

At the end of the reflux period approximately 300 ml. of the ethanol was distilled. To the residual mixture was added 500 ml. of 10% aqueous sodium hydroxide solution plus 250 ml. of water. The ethyl ester of the 3-ethoxy-2-naphthoic acid was then saponified directly without isolation over a four-hour period until a clear solution was obtained. The alkaline solution was permitted to cool and was then acidified by the addition of excess 37% hydrochloric acid (approx. 300 ml.). The warm mixture was stirred over a period of one hour and the yellow solid which formed was filtered, washed well with water, and dried. The crude product was recrystallized from 500 ml. of aqueous isopropyl alcohol (4 parts by volume of alcohol to 1 part of water). More product was obtained by concentrating the filtrates. Yield of 3-ethoxy-2-naphthoic acid, melting 119–123° was 127.8 Gm. (*ca.* 59%). Further purification of the acid was effected by recrystallization from about 2 parts w/v of 90–95% ethyl or isopropyl alcohol, using decolorizing carbon to remove some of the color. A purified sample of 3-ethoxy-2-naphthoic acid melted 123.6–125.5°. Wilke (23) reported 124°.

Anal.—Calcd. for $C_{13}H_{12}O_3$: Neut. Eq. 216. Found: Neut. Eq. 216.

3-Butoxy-2-naphthoic Acid, III.—Following essentially the same procedure described for compound II the butyl ether of 3-hydroxy-2-naphthoic acid was prepared, using 248 Gm. (1.35 moles) of butyl iodide in place of the ethyl iodide. In two experiments the average yield of 3-butoxy-2-naphthoic acid, melting 78–85°, was about 61%. Crude 3-butoxy-2-naphthoic acid was recrystallized in the same manner as described for the corresponding ethoxy analog. A purified sample melted 84.4–87.5°.

Anal.—Calcd. for $C_{15}H_{16}O_3$: Neut. Eq. 244. Found: Neut. Eq. 243.

Synthesis of the Acid Chlorides

3-Hydroxy-2-naphthoyl Chloride, IV, (24, 25).—The procedure used for the preparation of the acid chloride was essentially the same as that described by procedures given in the references. Yield, 61.8% of the theoretical; m. p. 96–97°, agreeing closely with the melting point values (96°, 94.5°) given in the literature (24, 25).

3-Ethoxy-2-naphthoyl Chloride, V.—Using the same procedure as described for compound IV, 112 Gm. (0.52 mole) of 3-ethoxy-2-naphthoic acid (II) was treated with 50 ml. of thionyl chloride in 500 ml. of ligroin by refluxing for five hours. After chilling well, the product was filtered and dried; the crude acid chloride weighed 101 Gm. (83% yield) and was used as an intermediate without further purification. These acid chlorides are relatively unstable and attempts to purify them by recrystallization from solvents were not too successful because of the rapid decomposition involved. Hill, *et al.*, (8), also prepared this compound (V). Isolation of the 3-alkoxy-2-naphthoyl chlorides was found to be unnecessary and in most cases it was more convenient and feasible to prepare the 3-ethoxy- or 3-butoxy-2-naphthoyl chloride, required for reaction with the amino-alcohols and amino-alkylamines, *in situ* in a suitable solvent (e.g. petroleum ether, benzene, $CHCl_3$).

Synthesis of the Esters (Series A)

Procedure A.—All of the esters reported in this paper were prepared essentially in the same manner as procedures A or B, which are modifications of general methods described by Robinson (8) and Cope (6) and are illustrated as follows:

2-Diethylaminoethyl 3-hydroxy-2-naphthoate (Hydrochloride), VI.—A solution of 5.9 Gm. (0.05 mole) of 2-diethylamino-ethanol in 30 ml. of chloroform was placed in a 100-ml. Erlenmeyer flask and was saturated with HCl gas under cooling to form the hydrochloride of the diethylaminoethanol. This solution was added to a second solution containing 10 Gm. (0.05 mole) of 3-hydroxy-2-naphthoyl chloride in 50 ml. of chloroform. The reaction mixture was heated under reflux in a 250-ml. single-necked, round-bottomed flask at 50–60° on a water bath for about eight hours. The product separated as white crystalline needles, which were filtered, washed with cold chloroform and ether and then dried. Concentrating the filtrate gave more product. The overall yield of the ester hydrochloride was 65.0% (10.4 Gm.). The compound melted at 184 to 184.6° and tended to sublime above 165°. It was found to be fairly soluble in water, slightly soluble in cold chloroform and cold ethanol, and insoluble in acetone and ether. The ester is soluble in hot chloroform and hot ethanol and can be recrystallized from either.

Anal.—Calcd. for $C_{17}H_{22}O_3NCl$: C, 63.2; H, 6.8; N, 4.3; Cl, 11.0. Found: C, 62.3; H, 6.2; N, 4.3; Cl, 11.2.

Procedure B.—To 0.2 mole of the amino-alcohol in 100 ml. of chloroform was added 0.21 mole of the 3-hydroxy-2-naphthoyl chloride in small portions. After the final addition, the solution was refluxed one hour. It was then filtered and evaporated to a syrup. Addition of a little isopropyl alcohol induced crystallization. It was then filtered and recrystallized.

Procedure C.—An alternate procedure for the preparation of these dialkylaminoalkyl esters is a modification of a method described by Burtner and Cusic (7), which is again illustrated with the synthesis of Compound VI.

To 5.5 Gm. (0.04 mole) of the free base of 2-diethylaminoethyl chloride (prepared from the commercially available hydrochloride salt by making alkaline and extracting with benzene) dissolved in 25 ml. of isopropyl alcohol, was added 7.5 Gm. (0.04 mole) of 3-hydroxy-2-naphthoic acid (I) in 25 ml. of isopropyl alcohol. The mixture was refluxed for one hour. At the end of this interval white crystals separated from the solution. The product was filtered and crystallized from 600 ml. of ethanol. The purified material melted at 183.5 to 184.3°. Yield, 9.4 Gm. (72.6%).

Anal.—Calcd. for $C_{17}H_{22}ClNO_3$: N, 4.32; Cl, 10.97. Found: N, 4.28; Cl, 10.88.

Synthesis of the Amides (Series B)

Procedure A.—This procedure is illustrated with the preparation of compound XIX.

TABLE V.—RELATIVE ANESTHETIC ACTIVITY AND TOXICITY OF THE DIALKYLAMINOALKYL ESTERS (SERIES A)

No.	Compound	Relative Anesthetic Activity,[a] Rated Numerically in Decreasing Order of Effectiveness: "1" = Most Active	Relative Toxicity,[b] Rated Numerically in Increasing Order of Toxicity: "1" = Least Toxic		
			Subcutaneous	Intraperitoneal	Intravenous
VII	3-Diethylaminopropyl 3-hydroxy-2-naphthoate hydrochloride	1	2	3	3
XII	3-Dibutylaminopropyl 3-hydroxy 2-naphthoate hydrochloride	2	..	..	..
X	2-Diethylaminopropyl 3-hydroxy-2-naphthoate hydrochloride	3	1	1	2
	Xylocaine[c]-epinephrine (2% solution)	4	5	5	4
XI	2-Dibutylaminoethyl 3-hydroxy-2-naphthoate hydrochloride	5	..	..	..
VI	2-Diethylaminoethyl 3-hydroxy-2-naphthoate hydrochloride	6	3	2	..
	Procaine hydrochloride	7	4	4	1

[a] The anesthetic activity was tested according to the rabbit corneal method of Sollomann (11).
[b] The relative toxicity studies were run on mice. The doses ranged from 9 to 25 mg. per 20 Gm. and the mortality at seventy-two hours was noted. Standard methods and techniques were followed.
[c] Xylocaine = α-diethylamino-2,6-aceto-xylidide hydrochloride.

TABLE VI.—RELATIVE ANESTHETIC ACTIVITY AND TOXICITY OF THE AMINOALKYL AMIDES (SERIES B)

No.	Compound	Rabbit Corneal Surface Anesthesia[a] Duration, Min.	Retrobulbar Block Test in Guinea Pigs[b]		Toxicity Approx. LD_{50}[c], mg./Kg.
			Pupillary Failure to Respond to Light, Min.	Corneal Anesthesia, Min.	
XIX	N-(2-Diethylaminoethyl)-3-hydroxy-2-naphthoamide hydrochloride	28	18	10	600
XX	N-(3-Diethylaminopropyl)-3-hydroxy-2-naphthoamide hydrochloride	23	44	8	725
XXIII	N-(3-Dimethylaminopropyl)-3-hydroxy-2-naphthoamide (acetate)	3	..	..	..
XXIV	N-(3-Isopropylaminopropyl)-3-hydroxy-2-naphthoamide hydrochloride	17	..	..	..
XXV	N-(2-Diethylaminoethyl)-3-ethoxy-2-naphthoamide hydrochloride	32	28	22	475
XXVI	N-(3-Diethylaminopropyl)-3-ethoxy-2-naphthoamide (acetate)	41	52	30	165
XXVII	N-(2-Diethylaminoethyl)-3-butoxy-2-naphthoamide (acetate)	58	90	60	130
XXVIII	N-(3-Diethylaminopropyl)-3-butoxy-2-naphthoamide (acetate)	40	57	27	125
XXX	N-(3-Dimethylaminopropyl)-3-butoxy-2-naphthoamide (acetate)	Effective but irritating	65	67	175
	Procaine hydrochloride	1	17	11	425
	Nupercaine[d]	..	..	..	10
	Cocaine	..	..	..	125

[a] See footnote *a* in Table V; 0.05% aqueous solution used for this test.
[b] This test was carried out by the method of Ferguson and Alkenhead (12). For these tests 0.2 cc. of a 0.1% solution was injected through the eyelid into the back of the eye.
[c] The approximate LD_{50} factor is that quantity expressed in mg./Kg. of mouse which will kill 50% of the mice injected subcutaneously.
[d] Nupercaine = the 2-diethylaminoethyl amide of 2-butoxy-cinchoninic acid.

N - (2 - Diethylaminoethyl) - 3 - hydroxy - 2-naphthoamide Hydrochloride, XIX.—Forty-six grams (0.4 mole) of 2-diethylaminoethylamine was dissolved in 500 ml. of purified benzene. To the benzene solution was added portionwise 80 Gm. (0.39 mole) of 3-hydroxy-2-naphthoyl chloride (II) while stirring. The resulting reaction mixture was heated gently under reflux for one hour. At the end of this interval the precipitate, which at first separated as an oil, turned crystalline. Purification of the product was effected by adding 500 ml. of isopropyl alcohol to the benzene suspension, heating the mixture until amost complete dissolution was obtained, filtering, and permitting the amide to crystallize from the isopropyl alcohol-benzene solution. The crystals were filtered and dried. More product was ob-

TABLE VII.—PRIMARY BACTERIOLOGICAL SCREENING TESTS ON THE QUATERNARY AMMONIUM SALTS[a] (SERIES C AND D)

		Bacteriostatic at					Fungistatic at				
No.	Compound	*S. aureus* 209-P	*B. subtilis* No. 6633	*E. coli* No. 9637	*M. phlei* No. 355	*C. albicans* No. 204	*A. niger* No. 337	*C. globosum* Q. M. 459	*A. oleracae* B. T.	*A. oryzae* No. 10196	*T. mentagrophytes* No. 9533
XXXI	2-Diethylaminoethyl-3-hydroxy-2-naphthoate methobromide	3	<3	<3	4	<3	<3	<3	3	<3	<3
XXXIII	3-Diethylaminopropyl-3-hydroxy-2-naphthoate methobromide	3	<3	<3	5	<3	<3	<3	3	<3	<3
XXXV	2-Diethylaminopropyl-3-hydroxy-2-naphthoate methobromide	3	<3	<3	5	<3	<3	<3	3	<3	<3
XXXIV	3-Diethylaminopropyl-3-hydroxy-2-naphthoate benzyl chloride	4	<3	<3	6	3	<3	3	3	<3	3
XXXVII	N-(2-Diethylaminoethyl)-3-hydroxy-2-naphthoamide methobromide	<3	<3	<3	4	<3	<3	<3	<3	<3	<3
XLI	N-(2-Diethylaminopropyl)-3-ethoxy-2-naphthoamide methobromide	<3	<3	<3	4	<3	<3	<3	<3	<3	<3
XLIV	N-(3-Diethylaminopropyl)-3-butoxy-2-naphthoamide methobromide	3	<3	<3	5	3	<3	<3	3	<3	<3
XLVI	N-(3-Dimethylaminopropyl)-3-butoxy-2-naphthoamide benzyl chloride	4	<3	<3	6	4	<3	3	3	<3	3
G 11	Bis(2-hydroxy-3,5,6-trichlorophenyl)-methane	6	6	3	6	4	<3	4	4	<3	4
G 4	Bis(2-hydroxy-5-chlorophenyl)-methane	6	3	<3	6	4	<3	5	4	3	5

[a] The antibacterial and antifungal tests were carried out by a plate streak method. The activity of the quaternary ammonium salts is compared with that of G-11 and G-4. The concentrations are expressed as the reciprocal of the logarithm of the dilution, e. g., 6 represents a dilution of 1:1,000,000.

tained by concentrating and chilling the filtrate. The pale yellowish-white crystalline compound was obtained in a 69% yield (87 Gm.); m. p. 156.5–157.6°. See Table II for analytical data.

Procedure B.—This procedure was a modification of procedure A.

To 23 Gm. of 2-diethylaminoethylamine in 200 ml. of chloroform was gradually added 40 Gm. of 3-hydroxy-2-naphthoyl chloride, keeping the solution just below the boiling point by the additions. The mixture was allowed to stand two days. It was then evaporated to half its volume and seeded. The pasty mass that formed was mixed with two volumes of isopropyl alcohol and chilled. The solid was removed by filtration, and recrystallized from isopropyl alcohol. Yield, 46 Gm. (70%); m. p. 155.5–158.5°.

Compounds XX, XXIV, and XXV were prepared by essentially the same methods described under procedures A and B.

Procedure C.—This procedure illustrates the preparation of a basic amide isolated and purified as the free base and is exemplified by the synthesis of compound XXVII.

N-(2-Diethylaminoethyl) 3-butoxy-2-naphthoamide (XXVII).—In 50 ml. of petroleum ether (b. p. 65–110°) was suspended 12 Gm. (0.05 mole) of 3-butoxy-2-naphthoic acid. To the suspension was added 7.6 Gm. (0.064 mole) of thionyl chlorides. The reaction mixture was refluxed under anhydrous conditions for five hours. At the end of this period complete dissolution was obtained and the solution was concentrated to one-half of its original volume with the aid of gentle heating and reduced pressure. After cooling the concentrate in an ice bath, 16 Gm. (0.138 mole) of 2-diethylaminoethylamine, dissolved in 160 ml. of benzene, was slowly added while stirring. The resulting mixture was refluxed for a three-hour period and then concentrated *in vacuo* to about one-fourth of its volume. On standing, crystalline needles separated. These were filtered and dried. More product was obtained by evaporating the filtrate and recrystallizing the oily residue from aqueous isopropyl alcohol. Total yield of product, melting 75–77°, 12.4 Gm. (72.5%).

Anal.—Calcd. for $C_{21}H_{32}O_3N_2$: C, 73.7; H, 8.8; N, 8.2; mol. wt., 342. Found: C, 73.7; H, 8.8; N, 8.1; mol. wt., 339.

Essentially the same procedure was used for compounds XXIII, XXVI, XXVIII, and XXX. In these cases the crude free basic amide, obtained by distilling off the solvent, was purified by dissolving it in dilute (10–20%) aqueous hydrochloric acid and precipitating it with 10–20% aqueous ammonia.

The solid amide was further purified by recrystallization from suitable solvents as indicated in Table II.

Synthesis of Organic Salts of the Basic Esters (A) and Amides (B)

Procedure A.—This procedure is illustrated by the preparation of compound XXI, which is an example of a crystalline organic salt isolated and purified by crystallization from a solvent.

N - (3 - Diethylaminopropyl) - 3 - hydroxy - 2-naphthoamide d-Tartrate (XXI).—The synthesis of the organic acid salt first required preparation of the free base of N-(3-diethylaminopropyl)-3-hydroxy-2-naphthoamide hydrochloride (XX). The hydrochloride XX was prepared by treating 150 Gm. (1.17 moles) of 3-diethylaminopropylamine dissolved in 1,300 ml. of benzene with 3-hydroxy 2-naphthoyl chloride (240 Gm.; 1.16 moles) by essentially the same procedures (A and B) described under the synthesis of the amides.

The aqueous solution of the hydrochloride salt was now treated with 300 Gm. (3.57 moles) of sodium bicarbonate to cause the separation of the base as a brown, oily lower layer, which was dissolved in 2 liters of benzene and the resulting solution was dried over anhydrous sodium sulfate. The solvent was removed by distillation *in vacuo* and the free base was obtained as the residue in the form of an orange-colored, viscous oil. Weight, 274 Gm. (78.7% yield).

With the aid of gentle warming in a water bath (at about 50°) 254 Gm. (0.85 mole) of N-(3-diethylaminopropyl)-3-hydroxy-2-naphthoamide was dissolved in 1,100 ml. of anhydrous ethanol and in similar manner 63.6 Gm. (0.424 mole) of *d*-tartaric acid was dissolved in 400 ml. of anhydrous ethanol. The two clear alcoholic solutions were mixed in a 5-liter three-necked, round-bottomed flask which was equipped for vacuum distillation and heated for *ca.* five minutes in the water bath at about 50°. At this point yellow crystals of the tartrate salt of N-(3-diethylaminopropyl) 3-hydroxy-2-naphthoamide began to separate. The mixture was permitted to stand for fifteen minutes and the first crop of crystals was filtered, washed with 100 ml. of cold ethanol, pressed dry as possible with the aid of the water suction pump, and then air-dried. Additional crops of product were obtained by concentrating the alcoholic filtrates. Total weight, 211 Gm. (67.13% yield). The yellow crystals melted at 156–157°. See Table II for analytical data.

Procedure B.—This procedure is illustrated by the preparation of the *d*-bitartrate (VIII) and *d*-tartrate (IX) salts of 3-diethylaminopropyl-3-hydroxy-2-naphthoate and analogous salts (see footnote *b* in Table I). It comprises mixing calculated quantities of the free base of the basic ester or amide and the organic acid in water to form a solution of the salt and then evaporating the water in some feasible manner such as codistillation with chloroform. The bitartrate of N-(3-diethylaminopropyl)-3-hydroxy-2-naphthoamide (XXII) was prepared in a similar manner using ethanol in place of the water. Distillation of the alcohol gave a taffy-like residue, which was converted to a pale yellow, somewhat sticky, hygroscopic solid by trituration with dry ether.

Synthesis of the Quaternary Ammonium Salts (Series C and D)

Procedure A.—This procedure represents the method used for preparing the methobromides of the basic esters and amides and is exemplified by the synthesis of compound XXXI.

2-Diethylaminoethyl 3-hydroxy-2-naphthoate Methobromide (XXXI).—The free base of 2-diethylaminoethyl 3-hydroxy-2-naphthoate (prepared essentially in the same manner as the free base of XX) (18 Gm., 0.063 mole) was placed in a Carius Pyrex combustion tube (19 x 25 x 615 mm.). After chilling the tube in a dry ice-methanol bath, an excess of previously chilled methyl bromide (approx. 14 ml.) was quickly poured into the tube, which was then sealed after thorough chilling. The charged tube was then placed in a calibrated, electrically-heated pipe furnace. The furnace was slowly heated up to a temperature of 100° and then held for four hours at a temperature range between 100–110°. The tube was permitted to cool slowly overnight in the furnace. After carefully breaking the seal, excess methyl bromide was allowed to distill off leaving the crude reaction product in the tube as a brown solid material. Working with various solvents, it was found the crude methobromide could be recrystallized from about 450 ml. of a boiling solvent consisting of isopropyl alcohol (90% v/v). The purified methobromide was obtained as a pale yellow crystalline product melting 169.1 to 170.8.° Yield, 18 Gm. (*ca.* 75%). See Table III for analytical data.

Procedure B.—This procedure describes the preparation of ethobromide and methiodide salts which are illustrated with the synthesis of compounds XXXVIII and XLII.

N - (2 - Diethylaminoethyl) - 3 - hydroxy - 2 - naphthoamide Ethobromide (XXXVIII).—N-(2-Diethylaminoethyl)-3-hydroxy-2-naphthoamide hydrochloride (XIX) (15 Gm., 0.0465 mole) was converted to the free base by the methods described previously using toluene as extraction solvent. The dried, filtered toluene extract was transferred to a 250-ml. round-bottomed flask. To the toluene solution was added excess ethyl bromide (15 ml.) and the mixture was refluxed over a twelve-hour period. During the reaction period the desired product separated from the solution as a lower oily layer. After permitting the reaction mixture to cool, the supernatant layer was decanted from the precipitated oil. The stiff oil was washed with 100 ml. of dry benzene and the wash was added to the toluene layer. Excess solvent and ethyl bromide were removed from the crude oily product by distillation *in vacuo* with the aid of a water bath and a water suction pump. During this operation the oily material was converted into a solid mass. It was found to be hygroscopic and difficult to recrystallize. After drying the product in a vacuum desiccator over sulfuric acid, it was found to soften at about 65° and to melt completely at 70°. Overall yield, 9 Gm. (49%). See Table IV for analytical data.

N - (2 - Dimethylaminoethyl) - 3 - butoxy - 2 - naphthoamide Methiodide (XLII).—N-(2-Diethylaminoethyl-3-butoxy-2-naphthoamide (free base) (XXVII) (1 Gm., 0.003 mole) was dissolved in 20 ml. of benzene. To the solution was added 1 ml. of methyl iodide. The reaction mixture was re-

fluxed six hours. During this period the crude methiodide separated from the solution as an oil which settled to the bottom of the flask. On standing at room temperature the oil gradually turned solid. The solid material was triturated with benzene, filtered and dried. It weighed 1 Gm. (69% yield) and melted 128–146°. It was recrystallized quantitatively by dissolving the solid in a small volume of ethanol and adding an excess of ether. Long, pale yellow needles melting 145.2 to 146.4° were obtained. See Table IV for analytical data.

Procedure C.—This procedure describes the preparation of benzyl chloride salts of the basic esters and amides and is exemplified by the synthesis of compounds XXXII and XLVI.

2 - (Diethylaminoethyl) - 3 - hydroxy - 2 - naphthoate Benzyl Chloride (XXXII).—Twenty-five grams (0.077 mole) of 2-diethylaminoethyl-3-hydroxy-2-naphthoate hydrochloride (VI) was dissolved in 2,500 ml. of water with the aid of gentle warming on a steam bath and the solution was made slightly alkaline by the addition of about 20 Gm. of sodium bicarbonate. The alkaline mixture was extracted with 3 one-liter portions of benzene; the extracts were combined, dried with anhydrous sodium sulfate, and filtered. The solvent was distilled at atmospheric pressure, leaving the free base of the amino-ester as a yellow oil. Weight, 19.5 Gm. (conversion, 88.6%).

Fifteen grams (0.0523 mole) of the free base were dissolved in 200 ml. of toluene. To this solution was added an excess (50 ml.) of benzyl chloride. The resulting solution was refluxed for a total of twenty-four hours. During and at the end of the reaction period no separation of the quaternary ammonium salt from the toluene solution was observed as in the case of some analogous compounds. Most of the solvent was then removed with the aid of water suction and gentle heating on a steam bath. To the concentrate was added a large excess of dry ether (*ca.* 500 ml.), which caused the product to separate out on standing as a somewhat gummy, grayish-white solid. It was reslurried and triturated with dry ether containing ammonia gas to eliminate traces of benzyl chloride odor. The white crystalline powder melted 150.0 to 151.1°. Yield, 8.7 Gm. (40.3%). See Table III for analytical data.

N - (3 - Dimethylaminopropyl) - 3 - butoxy - 2-naphthoamide Benzyl Chloride (XLVI).—Six grams (0.018 mole) of 3-dimethylaminopropyl-3-butoxy-2-naphthoamide (XXX) and 15 ml. of benzyl chloride were added to 100 ml. of toluene and the resulting solution was refluxed for sixteen hours. During the reaction period, the crude product precipitated from the solution as an insoluble oily layer. After permitting the reaction mixture to cool, the upper toluene layer was decanted and the viscous oil was washed with 50 ml. of cold benzene, which was added to the toluene layer. The oily precipitate was dissolved in 30 ml. of isopropyl alcohol and the solution was filtered. To the filtrate was added, while stirring, a large excess (2 liters) of a solvent consisting of equal volumes of dry ethyl acetate and ether. On standing, a solid product was obtained. It was filtered, washed with 25 ml. of dry ether, and air-dried. Further drying was effected over P_2O_5 in a vacuum pistol at 100°. The yield of the benzyl chloride salt, melting 103–105°, was 6.0 Gm. (72.2%). See Table IV for analytical data.

SUMMARY

1. In general, all of the basic esters and amides (Series A and B) reported were found to possess pronounced anesthetic activity. Of all the esters tested 3-diethylaminopropyl 3-hydroxy-2-naphthoate (as the hydrochloride) (VII), or bitartrate (VIII), was found to have the highest therapeutic index, producing anesthesia of longer duration than procaine. Members of the amide series which were found to be potent, fairly stable, and relatively nontoxic anesthetics were N-(2-diethylaminoethyl)-3-hydroxy-2-naphthoamide hydrochloride (XIX) andN-(3-diethylaminopropyl)-3-hydroxy-2-naphthoamide hydrochloride (XX), characterized as a crystalline *d*-tartrate (XXI). Another amide of interest was N - (2 - diethylaminoethyl)-3-butoxy-2-naphthoamide (XXVII), which although very active was found somewhat more toxic than procaine. Preliminary clinical reports (13–16) on these new local anesthetics appear to be of sufficient interest to warrant further study.

2. Compounds having antispasmodic activity which compared favorably with that of Trasentine, Panparnit, and Syntropan were N-(3-diethylaminopropyl) - 3 - butoxy - 2-naphthoamide (XXVIII), N - (3 - diethylaminopropyl) - 3 - hydroxy-2-naphthoamide (XXX), N-(2-diethylaminoethyl) - 3 - hydroxy - 2 - naphthoamide benzyl chloride (XXXIX), N-(3-diethylaminopropyl) - 3 - butoxy - 2 - naphthoamide benzyl chloride (XLV), and 3-diethylaminopropyl-3-butoxy-2-naphthoate benzyl chloride (XXX VI)

3. None of the compounds demonstrated sufficient antihistaminic activity to warrant further study.

4. 3 - Diethylaminopropyl - 3 - hydroxy - 2 - naphthoate benzyl chloride (XXXIV) and N-(3 - dimethylaminopropyl) - 3 - butoxy - 2 - naphthoamide benzyl chloride (XLVI) were found to be the most effective antibacterial agents of members of series A, B, C, and D tested, being bacteriostatic against four test organisms at dilutions of 1:10,000 both in the presence and absence of blood in the media. The 3- and 2-diethylaminopropyl esters of 3-hydroxy-2-naphthoic acid also exhibited some antibacterial effect. None of the compounds displayed any appreciable amount of antifungal activity.

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The Pharmacologic Actions of the Bronchodilator Drug, Isoetharine*[1]

By A. M. LANDS, F. P. LUDUENA, J. O. HOPPE, and IRENE H. OYEN

Isoetharine (Dilabron) is 1-(3,4-dihydroxyphenyl)-2-isopropylamino-1-butanol HCl. This drug is a potent bronchodilator agent, about one-half as effective as isoproterenol (Isuprel) but somewhat more effective than epinephrine. When combined with phenylephrine (Neo-Synephrine) and thenyldiamine (Thenfadil) some synergistic action was demonstrable. Dilabron has a comparatively low toxicity. Dilabron is absorbed readily following oral administration but this is usually associated with tachycardia. This can be prevented by enteric coating of the drug-containing tablets. In the experiments described here, the mean response in dogs medicated with enteric coated tablets was delayed more than fivefold over that obtained with uncoated tablets or gelatin capsules. These data appear to agree well with reported clinical results.

PREVIOUS RESEARCH carried out in our laboratories has disclosed enhanced bronchodilator action in the N-alkyl analogs of ethylnorepinephrine. The N-isopropyl analog isoetharine (Dilabron, Win 3046) is about thirty times more potent than the primary amine when activity is estimated in the guinea pig perfused lung, and thirty-five to fifty times more potent following parenteral administration to unanesthetized guinea pigs exposed to a histamine aerosol (1). Subsequent trials by Herschfus, *et al.* (2), in patients with chronic bronchial asthma in which the drug was administered as a 1.75 or 2.5% aerosolized solution, resulted in a marked increase in vital capacity with a minimum of side effects. However, cardiovascular side effects were marked and unpleasant after sublingual or oral administration (2, 3). Subsequent research has been carried out in an effort to develop pharmaceutical formulations in which these side effects are reduced while retaining essentially unchanged the therapeutic action of the compound. This communication describes the experimental results we have obtained with this new drug in various formulations.

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The authors are indebted to Miss Joan Hopper for technical assistance in carrying out the tests on unanesthetized guinea pigs, Mrs. Ellen Miller for the determinations of blood pressure and heart rate in unanesthetized dogs, and Mr. Leon Duprey for the acute toxicity determinations.

[1] Isoetharine is 1-(3,4-dihydroxyphenyl)-2-isopropylamino-1-butanol hydrochloride. It is identified commercially by the name Dilabron, a registered trademark of Geo. A. Breon and Co.

EXPERIMENTAL

The guinea-pig isolated lung preparation was perfused by the method described by Luduena, *et al.*

(4). In this procedure, guinea pigs weighing 140–200 Gm. are killed by a head blow, the lung removed with the trachea, and the lower lobes removed from the perfusion by ligation. The perfused lobes were scratched deeply to assure a good flow through the organ. Krebs-Henseleit solution was used in place of that of Van Dyke and Hastings, as described in the above method. Histamine was added to the perfusion fluid to make a concentration of 1:10 million (as base). All injections were made at a rate of flow through the lungs corresponding to a pressure in the calibrated vertical tube of 40 cm. water. The test drug was alternated with an injection of 0.2 γ epinephrine in order to dilate the bronchioles maximally before beginning each determination. The progressive bronchoconstriction induced by histamine was then interrupted by drug injection after the column of water had reached 40 cm. The dilatory effect was measured in cm. fall in this column, the height of the meniscus being plotted against time in minutes. The order of drug injection was changed in each of five experiments according to a randomized design.

Histamine induced bronchoconstriction in unanesthetized guinea pigs was determined by the method described in previous publications (5, 6). Histamine diphosphate, 0.2% in distilled water, was nebulized into a closed chamber to induce bronchoconstriction. The above concentration of histamine will cause asphyxial convulsions in 97% of exposed guinea pigs within twelve minutes. Bronchodilator drugs were added to this solution. The number of guinea pigs resistant to the action of histamine when combined with a bronchodilator drug and exposed to the mixture for twelve minutes was recorded, and a probit plot of the results used for the estimation of protective action (ED_{50}).

The protective action against intravenous histamine in guinea pigs was determined for Thenfadil[2] (thenyldiamine) and for Thenfadil in the Dilabron compound solution according to the procedure described by Lands, *et al.* (5). Guinea pigs, of both sexes, weighing between 300–400 Gm. were employed. The bronchodilator drug or formulation was injected subcutaneously thirty minutes prior to an intravenous injection of 0.5 mg./Kg. of histamine base ($2.8 \times LD_{50}$). Ten animals were used for each dose, with three or more doses being used in each determination.

Blood pressure changes were determined in anesthetized dogs (barbital sodium) arranged for the recording of carotid blood pressure in the usual manner. Heart rate changes were recorded simultaneously by means of the rate recorder described by Grumbach (7). All injections were made into the exposed femoral vein. Cardiovascular effects were determined in trained unanesthetized dogs, the pressure being obtained by auscultation and the pulse rate by palpation. The drugs were administered either subcutaneously or orally. Oral administration of Dilabron in plain gelatin capsule was compared with the enteric coated and uncoated tablets. Absorption of Dilabron occurs rapidly from solution along the gastrointestinal tract, so that when administered in solution to dogs by intubation, a response is noted within two to three minutes. Thus the appearance of tachycardia with large doses may be used to estimate the disintegration time of tablets containing the drug. In this investigation comparatively large doses of Dilabron (2–5 mg. total dose) have been used in order to evaluate the effect of tablet coating.

Acute intravenous toxicity was determined in Webster strain albino mice weighing 20 ± 2 Gm. as previously described by Hoppe, *et al.* (8). Groups of ten mice were medicated at each dose level with dosages arranged at equal logarithmic intervals. The LD_{50} ± standard error was calculated from the mortality at twenty-four hours.

Dilabron compound solution contains isoetharine [1-(3,4-dihydroxyphenyl)-2-isopropylamino-1-butanol HCl] 1%, thenyldiamine HCl (Thenfadil) 0.1%, and phenylephrine HCl (Neo-Synephrine) 0.25% in a suitable pharmaceutical formulation. Tablets contained Dilabron in a formulation which included bisulfite, lactose, starch, and magnesium stearate to which was applied a special layer of coating material to delay disintegration within the gastrointestinal tract. Uncoated tablets of the above were used for purposes of comparison.

RESULTS

Histamine Antagonism and Bronchodilator Action.—The results of previous investigations (1) have shown Dilabron (Win 3046) to be an effective antagonist of the bronchoconstrictor action of histamine in the guinea pig. In this action it is about one-half as potent as Isuprel. These studies now have been extended to include a comparison of the effects of Dilabron when formulated with an antihistaminic drug, Thenfadil, and a potent vasoconstrictor drug, Neo-Synephrine. The effects of Dilabron were compared with that of Dilabron combined with each component separately in the perfused guinea-pig lung. Results obtained are shown in Table I.

Dilabron is an effective bronchodilator drug with an activity of about one-half that of isoproterenol (Isuprel), but somewhat more effective than epinephrine (Lands, *et al.*, 1950). The addition of either Neo-Synephrine or Thenfadil as shown above increases bronchodilator action. The contribution of these drugs is further illustrated by the data which follow.

An effort has been made to determine the specific contribution of Thenfadil on histamine induced bronchoconstriction in guinea pigs exposed to a

TABLE I.—BRONCHODILATOR ACTION OF DILABRON AND DILABRON COMBINED WITH NEO-SYNEPHRINE OR THENFADIL IN THE GUINEA PIG ISOLATED PERFUSED LUNG

Dilabron,[a] 0.2 γ with			
Neo-Synephrine		Thenfadil	
Dose, γ	Effect, %	Dose, γ	Effect, %
0.050	110	0.020	96
0.165	114	0.066	127
0.50	142	0.200	134
1.65	154	0.660	148

[a] Dilabron response assigned a value of 100%. One injection of each dose of test drug was given and alternated with the standard. The order of injection was randomized in all experiments.

[2] Thenfadil (thenyldiamine HCl), Neo-Synephrine (phenylephrine HCl), and Isuprel (isoproterenol) are registered trademarks of Winthrop Laboratories.

nebulized solution of Dilabron. The method used has been described by Siegmund (6) who demonstrated potent protective bronchodilator action with Isuprel. A summary of the results obtained in the present investigation are shown in Table II. The dose-response curve obtained by plotting the per cent protection obtained against the log of the concentration gave an ED_{50} for Dilabron of about 0.01%. Similar values were obtained for Thenfadil. When the two drugs were combined they were more than additive, indicating some synergistic effect. Neo-Synephrine alone did not protect guinea pigs against inhaled histamine nor was this observed when added to a threshold concentration of Dilabron. Thenfadil, as contained in the compound solution, appears most important for effective bronchodilator potentiation.

TABLE II.—THE PROTECTIVE EFFECT OF DILABRON AND DILABRON COMBINED WITH NEO-SYNEPHRINE OR THENFADIL IN THE GUINEA PIG EXPOSED TO A HISTAMINE MIST

Drug	Concentration, %	Number of Animals	Protection
Dilabron	0.003	40	23
	0.010	46	57
	0.030	24	71
	0.100	26	92
Thenfadil	0.001	16	12
	0.003	26	27
	0.010	16	45
	0.030	16	81
Neo-Synephrine	0.250	10	0
Neo-Synephrine + Dilabron	0.100 0.003	20	5
Dilabron + Thenfadil	0.0015 0.0015	20	20
	0.0025 0.0025	20	75
	0.005 0.005	30	96
Dilabron + Thenfadil + Neo-Synephrine	0.010 0.001 0.0025 0.030	12	92
	0.003 0.0075	9	100

The protective action of Thenfadil administered subcutaneously, against intravenous histamine (2.8 × LD_{50}) was determined. Thenfadil alone has an ED_{50} of 0.013 ± 0.002 mg./Kg. When administered in the form of the compound solution, the protecting ED_{50} dose was reduced to 0.0069 ± 0.0007 mg./Kg. This value is one-half that obtained for Thenfadil administered in distilled water, indicating that Thenfadil is more effective in protecting against the toxic actions of histamine when combined in the Dilabron formulation.

Cardiovascular Actions.—Dilabron, like Isuprel, has important cardiovascular actions although these are less marked with the former (1, 2). The effects of the compound solution in comparison with Dilabron alone was determined by intravenous injection in barbitalized dogs, with results as shown in Table III. The average effect of the compound solution on blood pressure was somewhat less than that of Dilabron alone. Although the difference observed may not be significant for the small number of animals used here, there is a suggestion that the Neo-Synephrine content antagonizes somewhat the vasodilator action of Dilabron. This observation is strengthened by the results obtained in unanesthetized dogs injected subcutaneously with Dilabron and with the compound solution. In these experiments, the heart rate was markedly increased with the former, but with the latter there was less effect and a slower onset (Table IV). The Neo-Synephrine contained in the formulation may have delayed the rate of absorption of Dilabron into the general circulation. It is not improbable that a similar action would be obtained when the solution is applied to mucous membranes.

Enteric Coating of Dilabron Tablets for Delayed Oral Activity.—Previous clinical experience with Isuprel (9) has shown that oral administration caused a high incidence of side effects arising largely from the cardiovascular actions which this drug may induce. Absorption of Dilabron and Isuprel occurs readily following oral administration. This can be prevented by coating the drug-containing tablet with materials that will delay disintegration. Enteric coated tablets of Dilabron were administered to unanesthetized dogs and the results compared with those obtained when the drug was

TABLE III.—THE EFFECT OF DILABRON, NEO-SYNEPHRINE, AND DILABRON COMPOUND SOLUTION ON BLOOD PRESSURE AND HEART RATE OF THE ANESTHETIZED DOG

Drug	Dose I. V., γ/Kg.	Number Expt.	Av. Fall in Pressure,[a] Hg, mm.	Av. Fall in Pressure,[a] %	Av. Increase in Heart Rate, Beats/Min.
Dilabron	5	5	31 (20–42)	22 (16–38)	26 (12–48)
	10	3	43 (33–54)	26 (17–34)	28 (12–36)
Dilabron[b] + Neo-Synephrine	5	5	25 (18–42)	20 (9–46)	17 (12–36)
	1.5				
Dilabron[b] + Neo-Synephrine	10	3	30 (20–35)	16 (10–22)	20 (12–36)
	2.5				
Dilabron[b] compound solution	5	5	25 (19–41)	20 (14–45)	22 (12–36)
	10	2	31 (20–43)	18 (10–27)	36 (36–36)

[a] The number within the parenthesis gives the range of individual values from which the mean is computed.
[b] Dose of Dilabron in combination with Thenfadil and Neo-Synephrine.

TABLE IV.—THE EFFECT OF DILABRON ON BLOOD PRESSURE AND HEART RATE OF THE UNANESTHETIZED DOG AFTER SUBCUTANEOUS INJECTION

γ/Kg.	Number Expt.	Heart Rate, Beats/Min. Control	Increase	Onset	Duration	Blood Pressure, Hg, mm. Control (S/D)	Change	Duration
Dilabron								
20	3	73	77 (105%)	10–20	>133	113/79	+9/−6	..
40	1	110	110 (100%)	<10	>150	114/84	+26/−6	90
Comp. Sol.[a]								
20	3	77	45 (37%)	68–78	>200	115/83	+7/−4	..

[a] Dose of Dilabron in combination with Thenfadil and Neo-Synephrine.

administered in uncoated tablets or in plain gelatin capsules. Results are shown in Table V. The mean response in dogs medicated with enteric coated tablets was delayed more than fivefold over that obtained in dogs medicated with Dilabron in uncoated tablets or gelatin capsules. No effort has been made to compare the intensity of response in these experiments since quite large doses were given. Enteric coating has lengthened significantly the time of onset of Dilabron action.

Acute Toxicity of Dilabron.—The acute toxicity in mice has been determined for Dilabron and for the compound solution. A breakdown analysis of the formulation was carried out to determine the probable contribution of each major constituent. The method used for determining acute toxicity has been previously described (8). Data obtained are summarized in Table VI.

TABLE V.—THE EFFECT OF TABLET COATING (ENTERIC) ON THE ONSET TIME OF TACHYCARDIA IN UNANESTHETIZED DOGS

Dog No.	Onset Time, Min., of the Medication[a] Uncoated	Coated	Gelatin
1	30	140	...
2	25	210	..
3	20	165	..
4	30	240	30
5	50	75	35
6	105	150	..
7	25	345	..
8	10	>420	..
9	40	260	45
10	15	125	100
11	55	>120	..
12	20	225	..
Average	35	>206	..

[a] The dose administered was 5 mg. in the case of the coated and uncoated tablets. The dose administered in gelatin capsules was 2.34–2.70 mg. These large doses were selected in order to induce a marked tachycardia which would permit an estimate of the time of disintegration of the tablet. A dose of 5 mg. in solution administered by stomach tube caused tachycardia within three to four minutes.

Dilabron compound solution has a relatively low toxicity. The value of 1.7 ± 0.2 cc./Kg. of undiluted solution would correspond to a dose of approximately 120 cc. in a 70-Kg. human. When the individual toxicities of the ingredients of the compound solution were calculated from the LD_{50} values for the mixture, the greatest increase was contributed by Thenfadil and the least by Dilabron. When the toxicities of component parts of the mixture were analyzed by sequential deletion of one ingredient at a time, a significant reduction in toxicity was observed when the Neo-Synephrine HCl was omitted. The acute intravenous LD_{50} values for the mixtures containing Neo-Synephrine HCl plus Dilabron or Thenfadil were not significantly different from that of the compound solution.

DISCUSSION

Since the early demonstration of the effectiveness of parenterally administered epinephrine in the treatment of bronchial asthma, there has been a continuing search for a therapeutic agent that would provide equal or better symptomatic relief without the various side effects which characterize its action, that would be prolonged in action obviating the need for repeated medication at short intervals, and that could be administered by the patient while carrying on his accustomed activities. The use of epinephrine 1:100 by inhalation, as advocated by Graeser and Rowe (10), has become an established procedure for self-medication. However, this does not always prove satisfactory because of pulmonary edema which tends to limit penetration, and because of acquired resistance (epinephrine-fastness) which sometimes develops after prolonged administration.

The introduction of Isuprel into the medical armamentarium represented a distinct advance in that it was more potent than epinephrine, was effective in the epinephrine-fast patient, and could be

TABLE VI.—THE ACUTE INTRAVENOUS TOXICITY IN MICE OF DILABRON COMPOUND SOLUTION VERSUS THE MAJOR INGREDIENTS CONTAINED IN THE FORMULATION

Solution	Acute Intravenous LD_{50} ± S. E. Expressed in Terms of Undiluted Solution, cc./Kg.	Dilabron, mg./Kg.	Thenfadil, mg./Kg.	Neo-Synephrine HCl, mg./Kg.
Dilabron compound solution	1.7 ± 0.2[a]	17.0 ± 2.0	1.7 ± 0.2	4.2 ± 0.5
Dilabron 1.0% plus Thenfadil 0.1%	4.7 ± 0.4	47.0 ± 4.0	4.7 ± 0.4	...
Dilabron 1.0% plus Neo-Synephrine 0.25%	2.2 ± 0.28	22.0 ± 2.8	...	5.5 ± 0.7
Thenfadil 0.1% plus Neo-Synephrine 0.25%	2.45 ± 0.59	...	2.45 ± 0.59	6.1 ± 1.5
Individual components		57.0 ± 2.0	12.2 ± 0.6	22.5 ± 10.1

[a] The values obtained for Dilabron compound solution in six separate assays varied between 1.53 ± 0.19 cc./Kg. and 2.05 ± 0.24 cc./Kg.

administered sublingually as well as by inhalation or injection (11, 12). Various N-alkyl analogs of noradrenaline have been synthetized (13, 14) and studied as bronchodilator agents (15–17). One of these, in which the secondary substituent on the nitrogen of noradrenaline is 1-methyl-2(3,4-methylenedioxyphenyl)ethyl (JB-251, Caytine) has been evaluated clinically (18, 19). Results obtained with the inhalation of a 0.5% solution were comparable to those obtained with epinephrine 1% or Isuprel 0.5%. However, oral administration left much to be desired in that a dose of 2.5 mg. caused side effects in 52% of trials (21 out of 40 patients), the commonest effects being palpitation, tachycardia, and excessive nervous system stimulation.

Dilabron (Neosuprel) was evaluated by Herschfus, *et al.* (2), who reported that in a group of 49 patients, 1 ml. of the 1.75% solution when aerosolized with oxygen, increased vital capacity from a premedication value of 2.8 L. (range 1.4–4.8) to an average of 3.35 L. (range 2.2–5.4) for an average improvement of 550 ml. Side effects were not observed in these studies. The further interesting point is made that six patients with hypertension as well as bronchial asthma in whom Isuprel regularly induced palpitation, did not experience this reaction with Dilabron. Dilabron has been reported to produce good bronchodilatation after sublingual administration of 10 mg. (20). The compound solution described in this communication includes Neo-Synephrine 0.25% to provide decongestive action. Clinical trials with this formulation by inhalation in 339 cases has provided symptomatic relief in 97%, with an incidence of side effect of only 5.3% (3).

Oral administration of Dilabron, as with Isuprel and Caytine, has been reported to cause a comparatively high incidence of side effects (3). A 20 mg. dose in a plain tablet caused palpitation and tachycardia in every trial (75 cases). However, results obtained with the 5 and 10-mg. doses in the form of an enteric coated tablet has been without important side effects in most patients (21). These results would be expected from the experimental results obtained in this investigation and shown in Table V.

SUMMARY

An experimental investigation has been carried out on the new bronchodilator drug, N-isopropyl ethylnorepinephrine (isoetharine, Dilabron, Win 3046). Dilabron is an effective antagonist of histamine-induced bronchoconstriction in the guinea pig. This action is enhanced when combined with the actions of the potent antihistaminic drug, thenyldiamine hydrochloride (Thenfadil).

Dilabron in a formulation with Neo-Synephrine increases the protective action of Thenfadil against toxic i. v. doses of histamine in guinea pigs. The increase in pulse rate which characterizes the subcutaneous administration of Dilabron to dogs is diminished by adding Neo-Synephrine and Thenfadil in the proportions of 1.0:0.25:0.1. Oral administration to dogs of Dilabron in enteric coated tablets significantly delays the onset of action.

Dilabron, either alone as the pure compound or in its combination with Thenfadil and Neo-Synephrine, has a comparatively low acute toxicity when administered to albino mice.

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Examination of the Physical Chemical Basis for the Isoniazid-*p*-Aminosalicylic Acid Combination*

By JEROME A. REINSTEIN† and TAKERU HIGUCHI

Conductance, solubility, and spectrophotometric data indicate that the isoniazid-PAS combination in solution may be described as an equilibrium system containing unionized molecules, their salt forms, and at least two complex forms. At the low concentrations in which it would be found in body tissues, the complex forms would be expected to be completely dissociated.

THERE ARE A NUMBER of commonly used pharmaceutical combinations the chemical nature of which has never been established. They are variously referred to as salts, double salts, addition compounds, mixtures, and complexes.

In a solution system a particular two component combination may be defined as one of the following: (*a*) Mixture—where there is no specific interaction between the components, (*b*) Salt—where the two components are present as completely dissociated ions having opposite charges, (*c*) Complex—where the constituents are associated so that they exist as a single entity; e. g., caffeine-benzoate ion, and (*d*) Part salt, part complex—where there is an equilibrium between the salt and complex forms.

The purpose of the work was to determine which of these designations best fits the isoniazid-PAS combination.

It is a physical chemical fact that the ions of a salt do not remain together in a dilute solution system but act as individual moieties. Since ions move independently in solution, the probability of both ions being together at the site of action in the body is very small. If a combination in solution is a salt, therefore, there is no apparent reason why it should be therapeutically better than any other salt form of the active component which has approximately the same solubility and toxicity. The pharmacist on this basis should be free to use the salt best suited for a particular pharmaceutical use. Different salts may have different rates of solution. This may change somewhat the rate of appearance in the blood, but should not change the therapeutic action.

On the other hand if the constituents of a combination formed a stable complex with each other, then there is a small but finite possibility of some difference between the therapeutic efficacy of this combination and another because the components may be associated in the body. It is evident that, other than on a solubility basis, a combination must have a very great complexing tendency in order to have marked therapeutic advantage.

The isoniazid-PAS (INH-PAS) combination was first reported in this country by Brodhage (1). The combination was formed by mixing colorless equimolar solutions of isoniazid and PAS. The solution immediately turned yellow and a yellow substance containing isoniazid and PAS in 1:1 molar ratio was crystallized out.[1] Using this yellow combination, Brodhage stated that "the PAS salt of isoniazid shows a tuberculostatic effect which seems often to exceed that of physical mixtures of PAS and isoniazid."

Several laboratories in Europe have studied this compound, but the evidence presented for its chemical nature has not been conclusive Charonnat and Boime (2) concluded that the combination was a salt and that the linkage of the two compounds was through the pyridine nitrogen of isoniazid. Their work did not prove, however, that the bond between PAS and isoniazid at that site was ionic. Wilder Smith and Wiederkehr (3) stated that "the salicylate radical is attached to the side chain and not to the pyridine nitrogen atom" since "substitution on the hydrazine side chain nitrogen atom in INH hinders salt formation." They did not report their experimental procedures so their results could not be evaluated.

Complex formation between isoniazid and PAS was thought to be a distinct possibility since compounds similar to PAS (e. g., PABA, salicylic acid) have been shown to complex with compounds having nucleophilic sites resembling those of isoniazid (e. g., methylated xanthines) (4).

RESULTS

Three methods of studying the interaction were employed: electrical conductance, solubility, and spectrophotometry.

Conductance Study.—Conductance measure-

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[1] The compound is presently being used clinically in Europe.

ments were used to determine the extent of ionic dissociation of the compound on the premise that total dissociation would indicate no complex formation.

According to Kohlrausch's law of independent migration of ions, each ion contributes a specific amount to the conductance of an electrolyte, irrespective of the nature of the other ion. Thus: conductance of $INH^+ + PAS^-$ = conductance of isoniazid hydrochloride + conductance of potassium *p*-aminosalicylate − conductance of potassium chloride; and (Conductance of INH-PAS/Cond. $INH^+ + Cond. PAS^-) \times 100$ = per cent ionization of INH-PAS.

From the data in Table I, the per cent ionization of INH-PAS = $\{(a)/[(b) + (c) - (d)]\} \times 100$ $(159/199) \times 100$; 80% ± 5%.

TABLE I.—CONDUCTANCE MEASUREMENTS[a] FOR CALCULATION OF DEGREE OF IONIZATION OF INH-PAS

Solution	pH	Spec. Cond. (k/R) in Ohms^{-1} cm.$^{-1}$ × 10^6	Spec. Cond. Corrected for Cond. of (H^+) × 10^6
(*a*) 75 ml. of 0.005 *M* INH-PAS	3.80	215	159
(*b*) 75 ml. of 0.005 *M* INH plus 0.76 ml. of 0.2185 *M* HCl	3.80	324	268
(*c*) 75 ml. of 0.005 *M* PAS plus 0.45 ml. of 0.2214 *M* KOH	3.80	270	214
(*d*) 75 ml. of 0.001771 *M* KCl	...	283	283

[a] At 25° ± 0.1°.

The method of calculation of the strength of KCl used for solution (*d*) in Table I was as follows: molarity of K^+ in solution (*c*) = $[(0.45 \times 0.2214)/1000] \times (1000/75) = 0.001328$; molarity of Cl^- in solution (*b*) = $[(0.76 \times 0.2185)/1000] \times (1000/75) = 0.002214$; average = 0.001771 molar.

Subtracting the conductance of a 0.001771-molar potassium chloride solution from the sum of the conductances of (*b*) and (*c*) leads to an error of only 0.47% as compared with subtracting the sum of the conductances of 0.001328 molar potassium ion and 0.002214 molar chloride ion. The following calculation shows this to be true:

Specific conductance = molarity × molar conductance × 10^{-3}; specific conductance of 0.001328 *M* $K^+ = 0.001328 \times 73.52 \times 10^{-3} = 97.63 \times 10^{-6}$ ohm^{-1} cm.$^{-1}$; specific conductance of 0.002214 *M* $Cl^- = 0.002214 \times 76.34 \times 10^{-3} = 169.02 \times 10^{-6}$; specific conductance of K^+ plus $Cl^- = 266.65 \times 10^{-6}$; specific conductance of 0.001771 *M* KCl = $0.001771 \times 149.86 \times 10^{-3} = 265.40 \times 10^{-6}$; difference = 1.25×10^{-6}.

Therefore the per cent error is: $[(1.25 \times 10^{-6})/(265.40 \times 10^{-6})] \times 100 = 0.47\%$. This is within the error of the experiment.

The conductance of the (H^+) in solutions (*a*), (*b*), and (*c*) of Table I was subtracted from the specific conductance to give the corrected value. Specific conductance of 1.59×10^{-4} *M* H^+ = molarity × molar conductance × $10^{-3} = 1.59 \times 10^{-4} \times 350 \times 10^{-3} = 56 \times 10^{-6}$ ohm^{-1} cm.$^{-1}$.

Solubility Study.—The solubility study, which was similar to that described by Higuchi and Zuck (5), yielded the results shown in Fig. 1. Along the plateau from point *A* to point *B* a yellow substance precipitated. From points *B* to *C*, PAS continued to be precipitated from the solution combined with INH, and since there was no solid PAS to replace it, the total PAS in solution went down. From point *C* on it appeared that more soluble species of complex containing a higher ratio of INH to PAS predominated. If the only species in solution were the 1:1 combination, the concentration of PAS in solution after point *B* would have gradually fallen to the value of the solubility of the combination. Since it fell only slightly before starting to rise again, this indicated that there were other species in solution which were more soluble than the 1:1 species. At 0.140 molar isoniazid, the concentration of acid seemed to be too high. This higher value appeared to be due to a supersaturation effect. The curve was of the same form as those of Higuchi and co-workers (4, 5) in their demonstration of complex formation in similar compounds.

Stoichiometry of the Precipitated Complex (from Fig. 1).—Total PAS in the system = $(1.000/153.1) \times 20 = 0.131$ mole/liter. Total PAS in solution at point *A* = 0.037. Therefore 0.131 − 0.037 = 0.094 mole of PAS was left undissolved at point *A*. At point *B* all the excess undissolved PAS had been used up; that is, from *A* to *B*, the addition of 0.214 − 0.120 = 0.094 mole of isoniazid caused the precipitation of the undissolved 0.094 mole of PAS. Therefore the molar ratio of INH to PAS in the precipitated form was 0.094 to 0.094, or 1:1.

At pH 3.8, there are significant amounts of both the unionized and salt forms of INH and PAS. These forms could enter into a 1:1 complex in three different ways. These possible combinations are:

(1) $INH + PAS \rightleftharpoons INH - PAS$

(2) $INH + PAS^- \rightleftharpoons INH - PAS^-$

(3) $INH^+ + PAS \rightleftharpoons INH^+ - PAS$

From the data in this paper it was not possible to separate out these equilibria and to calculate stability constants for each one.

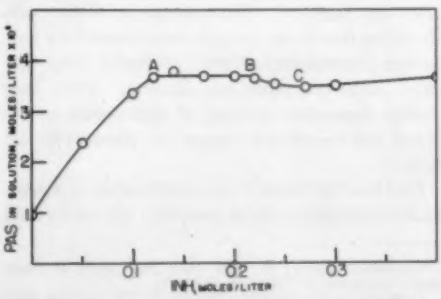

Fig. 1.—Interaction between INH and PAS.

Spectrophotometric Study.—The ultraviolet spectrophotometric study indicated strongly that the interaction between INH and PAS, in solutions of sufficient concentration, was at least partially complex formation, since the absorbance of the solution of both components (Fig. 3) showed marked deviations from the sum of the absorbances of its constituents. For example, at 375 mμ PAS did not absorb and INH absorbed only slightly; however, INH with PAS absorbed strongly.

If the combination were weakly bound in solution, it would be expected that the absorbance of a very dilute solution containing both INH and PAS would not differ from the sum of the absorbances of the PAS and INH separately since the complex would be completely dissociated in dilute solution. This is what happened when all constituents were at 10^{-4} molar concentration (Fig. 2).

At 375 mμ the absorbances of some of the compounds measured lent themselves to the calculation of an overall stability constant for the complex, since the absorbance of a species in solution is proportional to its concentration. Using simultaneous equations the stability constants in Table II were calculated. These constants, while of the same order of magnitude as those obtained from the solubility study, showed even poorer precision. This poor precision is attributed to the many different species in solution and the difficulty in

TABLE II.—STABILITY CONSTANTS CALCULATED FROM FIG. 3

Stability Constant	Solutions
42	INH = 0.1 M, PAS = {0.01 *M*, 0.02
62	PAS = 0.01, INH = {0.01, 0.025
74	PAS = 0.01, INH = {0.05, 0.075
142	PAS = 0.01, INH = {0.075, 0.1
102	PAS = 0.01, INH = {0.1, 0.15

reading the absorbance of the system from the graph; a relatively small difference in the absorbance of the system made a large difference in the stability constant. Different species of complex in solution also probably account for the unequal ratio of the absorbances of the solutions at 425 and 450 mμ (Fig. 3).

It was noticed that when a small amount of PAS solution was added to a concentrated isoniazid solution, a deeper yellow color was produced than that obtained when equimolar amounts of the two substances were mixed. When a small amount of isoniazid solution was added to a large amount of PAS solution, no unusual color changes were observed. A possible explanation of the deeper yellow color in the former is that the high relative concentration of INH to that of PAS led to the predominance of a more highly colored species in solution, in which the ratio of INH to PAS was greater than 1:1 . This seemed reasonable on the basis of the curves obtained in Figs. 2 and 3.

The yellow color of the INH-PAS combination cannot be rigorously explained at this time, but

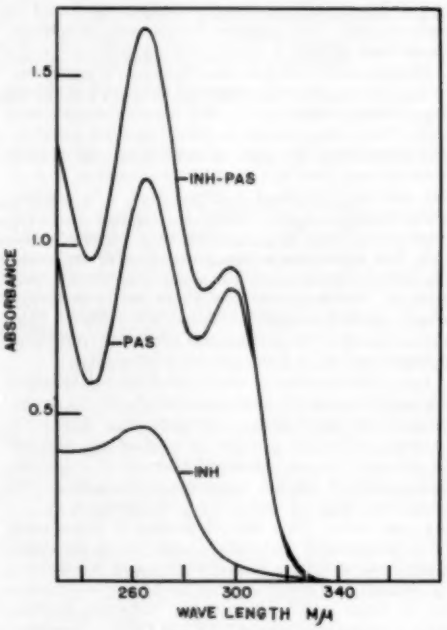

Fig. 2.—Spectra of INH, PAS and INH-PAS each at 10^{-4} molar and pH 4.64.

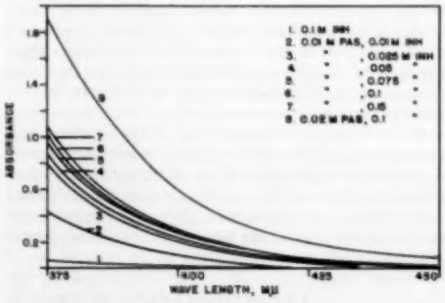

Fig. 3.—Absorption spectra of solutions containing varying quantities of INH and PAS.

it is unlikely that it is due to salt formation, since salification of PAS with potassium hydroxide and INH with hydrochloric acid did not produce a color. However, intensification of color with complex formation is known, as, for example, the picrate complexes and the yellow ascorbic acid-nicotinic acid complex. Perhaps a similar mechanism is responsible for the color associated with INH-PAS.

GENERAL DISCUSSION

From these studies it appeared that the combination in solution may be described as an equilibrium system containing unionized molecules, their salt forms, and at least two complex forms. However,

in low concentrations, as it is likely to be found in body tissues, the complex forms are completely dissociated.

Our physical chemical data were partly confirmed by a study made in Germany (6) where it was shown paper chromatographically that the combination was split into its components in dilute aqueous solution, and bacteriologically that "a mole to mole mixture of isoniazid and PAS has an activity similar to that of the unstable chemical combination." In another bacteriological study, Wilde (7) showed that the combination was not superior to a physical mixture, but with guinea pigs and mice (8) he found the yellow combination was superior to the physical mixture. Walker and co-workers (9) in Scotland, after a clinical evaluation, found no evidence that the isoniazid-PAS combination acts as a compound independent of its isoniazid and PAS content.

Both components of the INH-PAS combination are used separately and concurrently in the treatment of the same disease, tuberculosis. The PAS, in doses of 12 Gm. per day, is used as an adjuvant to 300 mg. per day isoniazid therapy to delay the appearance of strains resistant to isoniazid. The suggested dose of the INH-PAS complex is 700 mg. per day. This would contain a therapeutic dose of isoniazid, but only about $^1/_{40}$ of the usual daily dose of PAS. Wilder Smith and Wiederkehr (3) stated that their data showed the combination to be more active against the tubercle bacillus than physical mixtures of PAS and INH. However, the evidence for greater activity rested on bacteriological tests in which different strains were used in the tests with the complex than were used in the tests with the physical mixture of the two. Also in the low concentrations used in their study there would have been no difference between the very dilute solution of the complex and the very dilute solution of the physical mixture, since at that low concentration the combination is completely dissociated into its component parts.

There is some evidence that PAS administered simultaneously with INH may increase blood levels of INH by competition for acetylating agents (10). However, whether the combination is administered as the yellow complex or as a physical mixture of the two components should not affect the therapeutic action.

EXPERIMENTAL

The Conductance Study.—*Apparatus:* Leeds and Northrup Company Wheatstone bridge, RCA WO-88A oscilloscope, Hewlett Packard audio oscillator model 200C, conductance cell with cell constant = k = 0.317, Beckman model H 2 pH meter, constant temperature bath.

Materials: Isoniazid (Pfizer), *para*-aminosalicylic acid (Merck), both recrystallized; potassium hydroxide, hydrochloric acid, and potassium chloride, all reagent.

Procedure: The oscillator (with a 1,000 c. p. s. signal), oscilloscope and conductance cell were connected to the Wheatstone bridge. The oscilloscope was arranged so that a horizontal signal would indicate that the bridge was in balance. The resistance of each solution was determined and the conductance calculated.

Preparation of the Isoniazid-PAS Combination: 3.063 Gm. (0.02 mole) of PAS were dissolved in 80 ml. of anhydrous alcohol. Since the PAS solution was tan colored, about 0.5 Gm. of Norit was added and the solution filtered. The resulting colorless solution was mixed with a solution of 2.743 Gm. (0.02 mole) of isoniazid in 25 ml. of distilled water. The yellow solution obtained was warmed, evaporated in a stream of air to half volume, and put into a refrigerator overnight for crystallization. M. p. 140–141°, effervescence (Fisher-Johns melting point apparatus).

The Solubility Study.—*Procedure:* 1.000 Gm. of PAS was placed in each of twelve 125-ml. glass-stoppered bottles. Fifty milliliters of water plus varying quantities of isoniazid were added to each. The bottles were shaken at 30° for one and one-half hours; then they were seeded with a crystal of complex and shaken for one hour more. (Usually in a solubility study one equilibrates the samples for twelve to twenty-four hours, but since PAS slowly decomposes in solution, it was found that two and one-half hours of shaking produced near equilibrium without significant loss due to decarboxylation.) An aliquot portion of the solution in each bottle was then titrated with 0.1 N potassium hydroxide using bromothymol blue indicator.

The UV Spectrophotometric Study.—*Apparatus:* The spectra recorderd in Figs. 2 and 3 were obtained from a Cary Recording Spectrophotometer Model 11 MS, using water as the solvent at 25° ± 1°.

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Sulfaethylthiadiazole IV. Steady State Blood Concentration and Urinary Excretion Data Following Repeated Oral Doses*

By JOSEPH V. SWINTOSKY, AMEDEO BONDI, JR., and MANFORD J. ROBINSON

Experimental data are presented illustrating the use of a specific velocity constant for drug disappearance from the blood stream in the determination of dosage regimens. Equations are presented which permit estimation of the limits of steady state blood concentrations in adult human subjects receiving equal doses of sulfaethylthiadiazole at regular time intervals following a priming dose. These concepts are illustrated with experimental blood and urine data for subjects receiving fixed doses at one-, four-, and six-hour periods. The importance of these type concepts and data in pharmaceutical product development is discussed.

IN PREVIOUS PAPERS from these laboratories (1–4) it has been shown that sulfaethidole (sulfaethylthiadiazole or SETD) disappears from the blood and is excreted in the urine of man at approximately a first order rate for a considerable portion of a dose, following attainment of "psuedo steady state" diffusion equlibrium. The fact that rate of SETD disappearance from the tissues could be quantitated suggested study of "steady state" blood concentrations and urinary excretion rates in subjects who had received a sequence of identical oral doses of this drug at equally spaced time intervals. This concept is applicable to those drugs in which a direct correlation may be possible between blood concentrations and therapeutic efficacy. It is useful in aiding in design of dosage forms and regimens in pharmaceutical product development and evaluation.

EXPERIMENTAL

Part A.—Two Gm. SETD was administered orally in 0.5 Gm. hard gelatin capsules to four healthy young adult male subjects prior to breakfast and after control blood samples had been taken. Thereafter, each subject received one capsule containing 0.22 Gm. SETD every hour for eight consecutive hours. Blood samples were obtained by methods described previously (3, 4) at one, two, four, six, eight, and twelve hours after administration. Total urine collections were made for determination of drug output for four, eight, twelve, and twenty-four hours.

Part B.—In this study four similar subjects received a 2-Gm. oral dose of sulfaethylthiadiazole, as described above, followed by a 1-Gm. oral dose every six hours for sixty-six consecutive hours. A second group of four individuals received a 2-Gm. oral dose of SETD, followed by a 1-Gm. oral dose every four hours, for fifty-two consecutive hours. Blood samples were collected periodically throughout these periods. Total urine collections were made at consecutive twelve-hour periods, for ninety-six hours in the first study and for seventy-two hours in the other.

Analytical.—Concentrations of free as well as free plus conjugated (total) SETD in the blood and urine were determined by a spectrophotometric procedure (2, 5).

RESULTS AND DISCUSSION

Part A.—It had been observed previously that a single 2-Gm. oral dose in adult subjects provides peak blood concentrations near 10–12 mg. % and that the k_b for free or total SETD generally varies between 0.07 and 0.12 in these subjects (2). Therefore, one may calculate that a dose near 0.2 Gm. repeated every hour should maintain a blood concentration near the peak concentration of a 2-Gm. oral dose. Table I and Fig. 1 illustrate blood concentration data observed for 0.22-Gm. hourly doses following an initial 2-Gm. dose. The steady state blood concentrations were attained very rapidly for each of the subjects, and were maintained during the course of administration at about 10 to 14 mg. % as estimated by calculation. At twelve hours, the blood concentrations were beginning to subside.

In Table II the urinary excretion data for these subjects indicate a fairly uniform average excretion rate during the first 3 consecutive four-hour intervals, as would be predicted under approximately steady state conditions. About 80% of the total dose was excreted in twenty-four hours, with 1–7% SETD in the urine occurring as biotransformed product. The urinary data support previous findings (2, 3, 5). The composite blood and urine concentration data corroborate other statements (2–4, 6, 7, 9, 10) regarding the role of k or $t_{1/2}$ in a scientific approach to design of dosage forms and regimens.

Part B.—Standard clinical practice with sulfonamides and numerous other chemotherapeutic agents is to prescribe fixed doses at uniformly spaced time intervals. It should be possible to predict, for comparable subjects of similar weight, the course of blood concentrations during repeated oral admin-

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The authors wish to acknowledge the technical assistance of Drs. Elwood Foltz and Nicholas Viek in supervising the studies in human subjects, and Mrs. Betty Graves and Miss Barbara Vale for biochemical measurements.

This and previous papers of this series have been presented in part to the Fourth Canadian Conference on Pharmaceutical Research, Montreal, August 9, 1957.

TABLE I.—BLOOD CONCENTRATIONS OF SETD FOR FOUR MALE HUMAN SUBJECTS RECEIVING 2-GM. ORAL DOSES FOLLOWED BY 0.22 GM. HOURLY FOR EIGHT CONSECUTIVE HOURS

Subject	Age, yr.	Weight, Kg.	Hours	SETD Blood Concentrations Free, mg. %	SETD Blood Concentrations Total,[a] mg. %
Fs	22	75	0	0	0
			1	10.4	10.8
			2	11.7	12.3
			4	12.6	13.4
			6	11.6	12.2
			8	11.0	11.4
			12	9.2	9.4
Hn	22	67	0	0	0
			1	12.6	13.5
			2	13.0	13.2
			4	12.3	13.6
			6	12.2	13.8
			8	12.3	12.8
			12	9.2	9.5
Ja	22	85	0	0	0
			1	9.5	9.9
			2	12.8	13.2
			4	12.3	13.4
			6	11.8	12.3
			8	11.0	11.2
			12	8.7	9.3
Py	24	67	0	0	0
			1	12.8	13.4
			2	11.1	11.8
			4	11.9	12.8
			6	13.2	13.4
			8	13.2	13.8
			12	11.0	11.2

[a] Expressed as mg. equivalents of SETD.

TABLE II.—URINARY EXCRETION DATA FOR FOUR SUBJECTS RECEIVING 2-GM. ORAL DOSES OF SETD FOLLOWED BY 0.22 GM. HOURLY FOR EIGHT CONSECUTIVE HOURS

Subject	Hours	Urine Volumes, cc.	Urinary Excretion Free Drug, mg.	Urinary Excretion Total Drug,[a] mg.
Fs	0–4	965	618	705
	4–8	468	842	878
	8–12	812	828	853
	12–24	850	646	663
Hn	0–4	600	720	720
	4–8	420	1,067	1,071
	8–12	195	538	545
	12–24	555	777	810
Ja	0–4	158	506	545
	4–8	178	812	899
	8–12	180	734	756
	12–24	352	789	862
Py	0–4	717	516	531
	4–8	255	673	701
	8–12	180	594	599
	12–24	780	1,342	1,424
Average of the 4 subjects	0–4	...	590	625
	4–8	...	848	887
	8–12	...	673	688
	12–24	...	888	940

[a] Expressed as mg. equivalents of SETD.

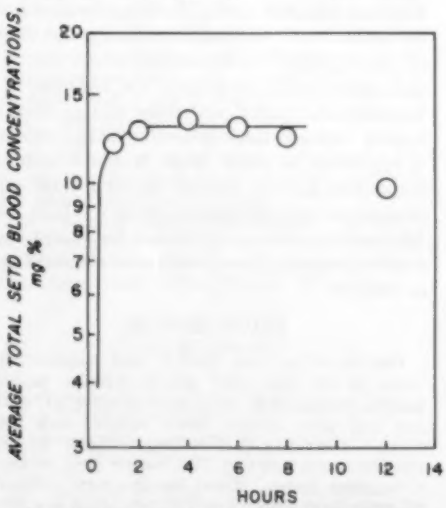

Fig. 1.—Average total SETD blood concentrations for four adult human subjects receiving 2 Gm. oral SETD initially and 0.22 Gm. each hour thereafter for eight hours.

istration when the elimination rate constant, k_b, is known, absorption rate is fairly consistent, and the relationship between the extrapolated maximum concentration, C_o, and the oral dose is known. Boxer, *et al.* (6), have presented calculations for the steady state plasma concentrations of streptomycin following repeated intramuscular injections of a fixed dose in human subjects.

The general equations illustrated by these workers for parenteral administration may be represented as follows: For a drug showing approximately first order rate of disappearance from the blood let C_o be the extrapolated blood concentration of a fixed dose at zero time as determined from a semilog graph paper plot.[1] Let k_b be the drug disappearance rate constant, and C the tissue concentration at time t. Then

$$\text{Log } C = -\frac{k_b t}{2.303} + \log C_o \qquad \text{(Eq. 1)}$$

or

$$C = C_o \cdot 10^{\frac{-k_b t}{2.303}} \qquad \text{(Eq. 1a)}$$

When T corresponds to a fixed time between doses, then

$$r = 10^{\frac{-k_b t}{2.303}}$$

and the blood concentration just prior to the second dose will be $C_o r$. Theoretically, just after the second dose it becomes $C_o + C_o r$. After the third dose it becomes $C_o + C_o r + C_o r^2$, and after n doses

$$C_n = C_o (1 + r + r^2 + \ldots . r^{n-1}) \qquad \text{(Eq. 2)}$$

Since r must be less than 1, C_n approaches an upper limit as n increases. By letting n approach infinity the sum of the geometric progression can be substituted in Eq. 2 to give this limit. Thus the maximum steady state tissue concentration is given by

[1] This C_o does not represent the administered dose divided by the effective volume of distribution. Its value is dependent in part on the rate of the drug absorption.

$$C \text{ max.} = \frac{C_o}{1 - r} \qquad \text{(Eq. 3)}$$

The minimum steady state tissue concentration is given by

$$C \text{ min.} = \frac{C_o r}{1 - r} \qquad \text{(Eq. 4)}$$

The methods of Boxer, *et al.*, should be applicable when SETD is administered orally, despite a finite time lag before a peak blood concentration is attained following each dose. The accuracy with which the time lag, T_1, can be estimated, corresponding to the beginning of exponential decrease of blood concentration after each dose, is a principal factor in the accuracy of the calculation of C max. The actual peak blood concentration following a single oral dose is given by $C_o \cdot 10^{(-k_b T_1)/2.303}$. The term $(-k_b T_1)/2.303$, which may be designated r_1, must be multiplied by C_o in Eq. 3 when applied to oral doses. Then, for an oral dosage regimen one may write

$$C \text{ max.} = \frac{C_o r_1}{1 - r} \qquad \text{(Eq. 5)}$$

and

$$C \text{ min.} = \frac{C_o r}{1 - r} \qquad \text{Eq. (6)}$$

The latter equation is the same as Eq. 4 where administration is parenteral; however, for equal doses in the same subjects, C_o does not have the same value by parenteral and oral administration.

Knowing that for this type of subject k_b varied generally between 0.07 and 0.12 when time is expressed in hours, and the C_o for total SETD is approximately 7 mg. % following a single 1-Gm. oral dose (2), using Eqs. 5 and 6 one may calculate that steady state total drug concentrations should lie between 11.5 and 25, and 6.5 and 18 mg. % for oral dosage regimens of 1 Gm. SETD every four or six hours, respectively. This is borne out in the experimental data of Figs. 2 and 3, where initial 2-Gm. doses were administered to hasten the approach to the steady state. In Figs. 2 and 3, the vertical distances between the circles denote the limits of the experimental blood concentrations observed for total SETD at the times indicated. Free drug concentrations were only slightly lower, as has been noted previously (2). The jagged lines connect average total SETD blood concentrations of the experimentally determined data and interpolate concentrations at other times. Interpolations are based on the assumptions that blood concentration peaks occur two hours after each dose and that k_b for such subjects is about 0.09 (2–4). The graphs indicate that the mean values of the experimental blood concentrations are fairly constant through the entire period, but they lie nearer the lower calculated limits. These data correspond closely to a k_b of 0.09–0.12% or a biologic half-life for SETD of six to eight hours.

The constancy of total SETD excretion pattern following establishment of diffusion equilibrium and steady state blood concentrations is attested to in the urinary excretion data of Fig. 4. Here average cumulative total SETD excreted is plotted *versus* time, at about twelve-hour intervals during

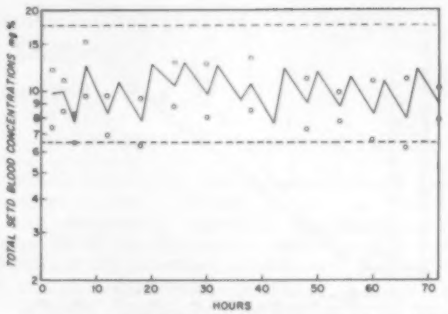

Fig. 2.—Total SETD blood concentration data for four adult human subjects receiving 2 Gm. SETD followed every six hours by a 1-Gm. dose for seventy-two hours. Broken lines indicate minimum and maximum limits calculated from Eqs. 5 and 6 where k_b is assumed to vary between limits of 0.07 and 0.12. Circles denote minimum and maximum experimental blood concentrations at times indicated.

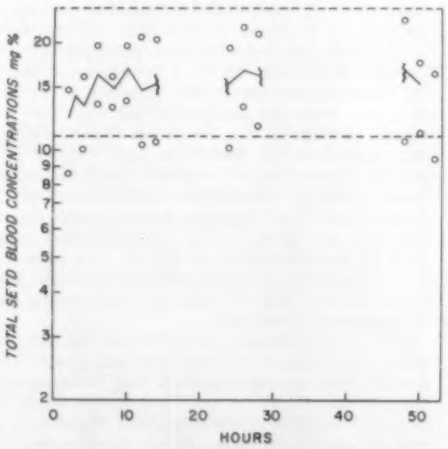

Fig. 3.—Total SETD blood concentration data for four adult human subjects receiving 2 Gm. SETD followed every four hours by a 1-Gm. dose for fifty-two hours. Broken lines indicate minimum and maximum limits calculated from Eqs. 5 and 6. Circles denote minimum and maximum experimental blood concentrations at times stated.

the period of administration. From the slopes of the lines during their straight line phases one may conveniently obtain a measure of absorption efficiency. When absorption and urinary excretion are 100% efficient one may calculate theoretical slopes of 0.250 and 0.166 for dosage regimens of 1 Gm. every four and six hours, respectively. Slopes actually observed in Fig. 4 are 0.24 and 0.16, indicating average absorption efficiency of 95% or greater for these subjects.[3] These results are further evidence of the predictability and efficiency of absorption of this drug from the gastrointestinal tract and its

[3] When urinary excretion is not the only method of elimination from the body, this technique may provide a useful approach to the accurate determination of the fraction of a dose eliminated via this route.

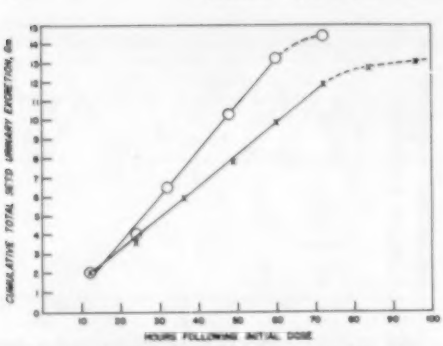

Fig. 4.—Average cumulative urinary excretion of total SETD in two groups of four adult human subjects corresponding to regimens of Figs. 2 and 3. Upper and lower lines correspond to 1 Gm. SETD administered each four and six hours, respectively.

excretion through the kidney. An average of 11% of the drug found in the urine was biotransformed; however, other studies have indicated an even lower per cent of biotransformation (2–5). These composite data and those reported previously (1–5,7) indicate that when SETD is administered orally the physician may expect very little loss of the dose in transit from the gastrointestinal tract into the tissues and finally into the urine. When the oral dose completes this transit, at least 85% of it is usually recoverable in the urine in its initial active form. None of the antibacterial sulfonamides in common use today appear to be recoverable to this extent in the urine in their original states other than the methyl homolog of SETD. For some sulfonamides (5, 8), as much as 50% of the initial dose is not recoverable in the urine in a form having useful antibacterial activity.

It is of both practical and academic interest that steady state blood concentration and urinary collection data may be estimated fairly satisfactorily by calculation. For a drug like SETD, where an apparent correlation exists between blood concentration and therapeutic efficacy, this theoretical approach facilitates a logical estimate of the amount of drug that should be incorporated into a dosage form, and its frequency of administration. Such information obtained in the early stages of industrial product development is valuable. It reduces the effort, time, and cost of empirical experimentation required to establish proper dosage strengths and regimens. Such information is also helpful in suggesting which dosage strengths and product formulas should be employed for stability studies. This, too, is helpful in getting stability study protocols established at the earliest possible times and in relieving analytical facilities of assay work on dosage forms of improper strengths.

SUMMARY

1. When four adult male human subjects received 2-Gm. SETD followed by 0.22 Gm. hourly through eight hours, steady state blood concentrations of total drug were maintained at 10–14 mg. % during the one- to eight-hour time interval and fairly uniform urinary excretion of drug resulted. These data verify that for SETD the velocity constant for elimination, determinable from blood concentration or urinary collection data, can be used to estimate the rate at which the drug must enter the tissues in order to maintain fairly constant blood or body concentrations.

2. Equations were developed to predict the "pseudo" steady state total SETD blood concentrations resulting from the administration of 1 Gm. every four or six hours. They apply to subjects typical of this study, assuming k_b to vary between 0.07 and 0.12, C_o of about 7 mg. %, and absorption occurring in two hours.

3. Experimental blood concentrations obtained from two groups of four adult male human subjects corresponded to the predictions of the equations.

4. Average cumulative total SETD urinary excretion plotted *versus* time was linear following establishment of diffusion equilibrium and steady state blood concentrations. The slopes of the lines indicate that at least 95% of the administered doses of SETD were absorbed.

5. The technique of plotting cumulative urinary excretion *versus* time may be useful for studies on other drugs where it is desired to obtain information regarding the fraction of drug excreted by the urinary route.

6. The results with SETD are further evidence of the predictability and efficiency of absorption of this drug from the gastrointestinal tract and its excretion through the kidney.

7. These and other data indicate that the physician may expect very little loss of the dose in transit through the body. In the average subject at least 85% of an oral dose is recoverable in the urine in its initial active form.

8. The role which the velocity constant for drug elimination plays in a scientific approach to establishing dosage regimens and formulas in product development is discussed.

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A Note on the Extraction and Fluorescence of Some Oxidation Products of Reserpine*

By EARL B. DECHENE

In a report by Banes, *et al.* (1), on the determination of reserpine in various types of pharmaceutical preparations, it is implied that oxidation products of reserpine, particularly 3-dehydroreserpine, would be estimated as reserpine by the fluorometric and colorimetric methods cited (2–4) in his report, unless correctives were applied.

All of these assay procedures require the extraction of reserpine from an acidified aqueous solution with chloroform, followed by washing the chloroform extracts with aqueous sodium bicarbonate. This note is to report our observations on the effectiveness of this extraction procedure in eliminating oxidation products of reserpine, and the extent of the interference, if present, in the determination of reserpine by a fluorometric method previously reported (2).

Separate solutions of isoreserpine, 3-dehydroreserpine, and reserpine treated with hydrogen peroxide or sulfuric acid, were submitted to the extraction procedure. The fluorescence and ultraviolet absorption spectrum of the extracts were measured, after removing the chloroform by heating with 5 *M* acetic acid.

Isoreserpine (5) was found to be completely removed from acid solutions by the extraction procedure. No measurable fluorescence was shown by the chloroform extracts before or after heating with hydrogen peroxide at a concentration of 1 mcg. in 10 ml. of solution.

3-Dehydroreserpine[1] (6) fluoresces strongly in acidified aqueous solutions. It was not completely extracted by chloroform from these solutions. Both the fluorescence and absorption at 380 mμ of the chloroform extracts were decreased after heating with hydrogen peroxide.

Oxidation products of reserpine produced by heating with hydrogen peroxide are extracted from acid solutions by chloroform. No fluorescence was shown by the extracts of solutions in which the alkaloid was completely oxidized. Solutions of reserpine in aqueous or glacial acetic acid when heated with hydrogen peroxide show an absorption spectrum with a maximum at 265 mμ only. The absence of a peak at 385 mμ indicated that 3-dehydroreserpine (6) is not present in these solutions. Solutions of reserpine treated at room temperature with hydrogen peroxide for four hours show an absorption peak at 383 mμ (7), and strong fluorescence. The chloroform extracts of these solutions show no measureable fluorescence and no significant absorption at 383 mμ.

Fluorometric values of the chloroform extracts of the solutions of reserpine heated with sulfuric acid were equal to about 20% of the theoretical reserpine content after heating with hydrogen peroxide. The solutions before extraction showed absorption maxima at 265 and 385 mμ as reported by McMullen (3). The absorbance at 385 mμ has been attributed to the presence of 3-dehydroreserpine (1). We found that chloroform extraction of these solutions removed about 40% of the materials absorbing at 385 mμ. The absorbance of the solutions of chloroform extracts decreased about 50% in the region of 385 mμ after heating with hydrogen peroxide. The data are summarized in Table I.

Table I.—Fluorometric Values and Absorbance Readings[a]

Material	Fluorometric Values (1 mcg./10 ml.) Solns. Before Extraction	Fluorometric Values (1 mcg./10 ml.) $CHCl_3$ Extracts	Fluorometric Values (1 mcg./10 ml.) $CHCl_3$ Extracts Heated with H_2O_2	Absorbance (100 mcg./10 ml.) Solns. Before Extraction	Absorbance (100 mcg./10 ml.) $CHCl_3$ Ext.	Absorbance (100 mcg./10 ml.) $CHCl_3$ Ext. Heated with H_2O_2	Absorbance (100 mcg./10 ml.) Maximum Found at mμ
Isoreserpine	0	0	0	0.192	0.192	0.205	265
3-Dehydroreserpine	50	32	22	0.277	0.135	0.071	380
Reserpine	3	3	37	0.280	0.285	0.532	265
Reserpine heated with H_2O_2	38	0	0	0.530	0.333	0.197	265
Reserpine heated with H_2SO_4	40	15	9	0.288	0.171	0.205	265
				0.248	0.092	0.050	383
Reserpine treated with H_2O_2 at R. T.	40	0	0	0.105	0.009	0	383

[a] All values are averages of 2 or more determinations.

Table II.—Results of Assays of Ampuls by Different Methods

	Nitrite Method, %	Nitrite Method Without Correction, %	Fluorometric Method, %
	59.7	80.6	59.2
	62.8	81.6	62.4
Av.	61.25	81.1	60.8
	81.2	100.4	77.32
	78.0	97.2	80.00
Av.	79.6	98.8	78.66
	80.90	96.04	80.00
	79.14	93.84	78.60
Av.	80.02	94.94	79.30

Solutions of reserpine containing, 50 mg. reserpine, 4% w/v benzyl alcohol, 4% w/v ethanol, 34% w/v glycerin, 39% w/v polyethylene glycol, water *q. s.* to 100 ml. were prepared. Five-ml. aliquots were placed in 10-ml. glass ampuls, sealed and auto-

* Received December 12, 1956, from The Research and Control Laboratories, Charles E. Frosst & Co., Montreal, Canada.

[1] We wish to thank Dr. F. L. Weisenborn of the Squibb Institute for Medical Research, for supplying this material.

claved for fifteen minutes at 125°. The reserpine content of the ampuls was determined by the nitrite method (1) and by the fluorometric method (2). The results of duplicate determination on three lots of ampuls are shown in Table II.

In the assays shown in Table II, the fluorometric method is measuring reserpine, in the presence of its oxidation products, as accurately as the nitrite method. The need for a blank correction in the nitrite method is clearly shown by the large differences in the corrected and uncorrected values.

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A Note on the Use of the Brookfield RVF Viscometer with Thickening Agent Dispersions*

By J. O. RUNIKIS,† NATHAN A. HALL, and L. WAIT RISING

DURING THE STUDY of the flow characteristics of various hydrophilic colloids used as thickening agents in pharmaceutical systems, it was found that correlation of viscosity measurements was difficult. Many of the data reported in the literature supplied by manufacturers and in pharmaceutical journals were not meaningful because of insufficient measurements or incomplete reporting of details. To improve the correlation of viscosity measurements and since the systems under examination were all non-Newtonian in nature, an investigation of the Brookfield RVF viscometer, a convenient instrument in common industrial use, was undertaken.

Single point determinations made with capillary or falling ball viscometers, although simple and reproducible, fail to give useful rheological information for non-Newtonian materials. This fact has been repeatedly emphasized (1–3). Rotational viscometer can give significant rheological data; however, difficulties such as expensive instrument construction, uncertain reproducibility of measurements, and involved calculation of viscosity have limited their use. To obtain comparable measurements on non-Newtonian materials a method must include a standardized preparation of samples, a standardized system of taking viscosity measurements, and a method of calculation based upon the flow behavior of the materials investigated.

The viscosity of non-Newtonian materials cannot be calculated directly from Brookfield data because of mathematical difficulties describing the dimensions of the spindle. Usually the viscosity is calculated from a calibration chart supplied by the manufacturer. Such a method of calculation is based, however, on the assumption that the calculated viscosity is independent of shear rate. This means that the Brookfield measurements are treated as single point measurements, and thus the advantages inherent in the rotational instrument are not used.

To make proper use of the Brookfield instrument with non-Newtonian systems, there is a need for an improved method of calculation. One such method has been proposed by R. L. Bowles, *et al.* (4). Bowles considers Brookfield viscosities as slopes connecting the actual flow curve with the origin. Two of these slopes suffice to approximate the true slope of the actual flow curve in the shear rate region for which the Brookfield viscosities are calculated. From this approximation the non-Newtonian viscosity and the yield value, assuming linearity of the actual flow curve, can be calculated. Bowles has demonstrated that the use of his equations improves the agreement of results between the Brookfield and Precision Interchemical Rotational viscometers.

The method of Bowles is in good accord with the basic principle that viscosity has to be calculated from a relationship in which shear stress and shear rate are included. But his assumption of linearity of the actual flow curves may introduce a certain measure of uncertainty. His method does not always distinguish between materials showing a yield value and those that do not. The viscosity is calculated from data of only one shear rate region, i. e., from only two torque readings, neglecting the remainder of the flow curve. When the limits of experimental accuracy are considered, the errors introduced are probably minor for most calculations; however, it seemed advisable to derive an alternate method of calculation which would take into account the entire flow data obtainable with the Brookfield instrument.

Such an alternate method can be derived for many viscous systems which show a regular change in their slopes and approach linearity on a log log plot. Many pharmaceutical thickening agents show this behavior. The derivation of the necessary equations is similar to that of E. K. Fischer who adapted the same idea for a modified Stormer type viscometer (1).

Viscosity can be expressed in the well-known manner as:

$$\text{Viscosity} = \frac{dF}{dS} \qquad \text{(Eq. 1)}$$

where F is the shear stress and S is the shear rate. Viscosity can thus be defined as the reciprocal of the slope of the tangent to the curve in a shear rate *vs.* shear stress plot, i. e., a plot of a flow curve. For the Brookfield viscometer the equation will assume the form:

* Received May 3, 1957, from the University of Washington, College of Pharmacy, Seattle 5.
† Fellow, American Foundation for Pharmaceutical Education.

$$\text{Viscosity} = K_B \frac{d \text{ scale reading}}{d \text{ r. p. m.}} \quad \text{(Eq. 2)}$$

where K_B is a constant that converts the ratio of the rate of rotation and the scale reading to viscosity in centipoises. The value of K_B is given by:

$$K_B = \text{spindle ratio} \times 20 \quad \text{(Eq. 3)}$$

where 20 is the highest rate of rotation available with the instrument. Equation 3 is valid because the scale of the Brookfield viscometer is so constructed that the scale readings equal centipoises when spindle #1 is used at 20 r. p. m. The other spindles will "read" higher viscosity in direct proportion to spindle #1. The spindle ratios for the 500 scale are: 1 (*S.* #1); 4, (*S.* #2); 10 (*S.* #3); 20 (*S.* #4); 40 (*S.* #5); 100 (*S.* #6); 400 (*S.* #7) where *S* denotes spindle.

For flow types whose flow curves pass through the origin and show logarithmic linearity (pseudoviscous or dilatant viscous flow types):[1]

$$\text{Scale reading} = A \text{ (r. p. m.)}^n \quad \text{(Eq. 4)}$$

where A is an unknown constant. Differentiating:

$$\frac{d \text{ (scale reading)}}{d \text{ (r. p. m.)}} = (A)(n)(\text{r. p. m.})^{n-1} \quad \text{(Eq. 5)}$$

or from Equations 2 and 4:

$$^2\text{Viscosity}_v = \frac{(K_B)(n)(\text{scale reading})}{(\text{r. p. m.})} \quad \text{(Eq. 6)}$$

For example, for spindle #3 the corresponding viscosity$_v$ value derived from Brookfield scale readings is:

$$\text{Viscosity}_v = \frac{(200)(n)(\text{scale reading})}{(\text{r. p. m.})} \quad \text{(Eq. 7)}$$

The value of n can be calculated most conveniently from a logarithmic plot of r. p. m. *vs.* scale reading, i. e., from the logarithmic flow curve plot, where n is the reciprocal of the slope.

To illustrate differences between the three methods of calculation, the viscosity of karaya gum, N. F. X, Hallmark Superior Grade XXXX,[3] 1%, was calculated using the Newtonian Brookfield conversion chart, the Bowles' equation:

$$\text{Corrected Viscosity}_{15} = 2V'_{20} - V'_{10} \quad \text{(Eq. 8)}$$

where V' is the Brookfield viscosity as calculated from the Newtonian conversion chart at rates of rotation indicated by the subscripts, and the proposed Eq. 7. The results were checked on separately prepared samples.

Table I shows that the indiscriminate use of the Brookfield conversion chart may introduce an error of more than 100%. The 640 c. p. s. obtained through the use of Bowles' correction represents viscosity at an approximated rate of rotation of 15 r. p. m. Recalculated at this rate of rotation (Eq. 4 and 7), the proposed n value method showed a viscosity of 630 c. p. s., a remarkably close agreement with Bowles' value.

TABLE I.—VISCOSITY OF KARAYA GUM 1%, CALCULATED BY THREE DIFFERENT METHODS

Brookfield 20 r. p. m.	Bowles' Eq. 8 $2V'_{20} - V'_{10}$	Eq. 7, 20 r. p. m.
1130 c. p. s.	640 c. p. s.	540 c. p. s.

DISCUSSION

The advantages of the described "n value method" of calculation are: The method does not require any new assumptions; the shape of the entire flow curve in the region of the measurement is taken into consideration; it permits the calculation of viscosity from a flow curve instead of through the use of a Newtonian calibration factor; the calculated n value can be used for the comparison of flow curves and serve as an indication of the complexity of structural arrangement in the liquid, because materials which show a reciprocal log log slope (the n value) of less than 1 are pseudoviscous, those of greater than 1 are dilatant viscous, and those with exactly 1 are Newtonian (1). For comparison of flow curves the method may be helpful because through it the pseudoviscous flow curves are characterized if either the n value and the logarithmic intercept (found through Eq. 4 or through extrapolation) or the n value and shear stress at any shear rate are known. Consequently the viscosity of pseudoviscous materials can be estimated at many shear rates.

The limitations of the n value method are: the method has no theoretical significance; the method cannot be applied to systems having a yield value and therefore can be used only for pseudoviscous and viscous dilatant materials; the rate of change of the slope of the flow curve has to be such that it can be expressed by a linear log log equation; if the alignment of the experimental points is not perfect, the n value has to be estimated from a plot which may introduce some uncertainty. These disadvantages are not serious because many materials of pharmaceutical importance show pseudoviscous flow and the precision of the measurements for non-Newtonian systems is generally not great.

Although the method was developed for the Brookfield RVF Viscometer, through only minor adaptations it should be readily utilizable with other models of the Brookfield design.

[1] The term pseudoviscous is used in preference to the more usual term, pseudoplastic, because it fits better the fundamental division of flow types in viscous and plastic flow categories (5). It also limits exactly the validity of Eq. 7. By definition, pseudoviscous materials are those that give nonlinear flow curves which are concave toward the rate of shear axis in the plot of a flow curve and do not show a yield value. The term pseudoplastic is reserved for materials with similar flow curves, but showing a yield value. This classification is in accord with that described by Doolittle (6).

[2] Viscosity$_v$ represents "viscous viscosity" and limits the validity of the method to the conditions described. It is used in analogy with the term "plastic viscosity." The term "apparent viscosity" customarily used, is of a less certain meaning.

[3] Stein Hall Company, 285 Madison Avenue, New York 17, N. Y.

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A Note on the Photosensitivity of Phenothiazine Derivatives*

By LOUIS J. RAVIN, LLOYD KENNON, and JOSEPH V. SWINTOSKY

DURING FORMULATION STUDIES it became evident that solutions of certain clinically useful substituted phenothiazines readily undergo chemical changes in the presence of light. This is shown visually by the formation of color. The literature indicates the susceptibility of phenothiazine and its derivatives to photochemical and oxidative changes (1–4); however, efforts to determine the rates of photochemical oxidation and to evaluate the course of photochemical changes in pharmaceutical systems of such compounds have not been reported. Stability studies utilizing exaggerated temperature conditions to screen formulations and predict shelf life of pharmaceutical products are well known. However, complementary techniques using higher light intensities and oxygen tensions for studying oxidative reactions in pharmaceutical systems are less well known. A program has been initiated to explore this field. This note presents some preliminary observations on two phenothiazine derivatives regarding oxygen uptake as measured by the Warburg[1] manometric technique. Photochemical changes were accelerated by exposing solutions in air to a light source.[2]

Chlorpromazine hydrochloride, SKF, and prochlorperazine ethanedisulfonate, SKF, were studied. In each case a 3-ml. sample of a 5% aqueous solution of the phenothiazine salt was placed in a Warburg flask containing 0.2 ml. of 10% KOH solution in the center cup. The reaction flasks and the thermobarometer flask were placed in a water bath set at 25°. The light source was mounted in a fixed position approximately four inches above the flasks. A cover was placed over the apparatus to exclude extraneous light. The flasks were shaken for ten minutes to establish thermal equilibrium prior to closing them, adjusting the manometers, and turning on the light. Oxygen uptake measurements were then started.

Figure 1 illustrates oxygen uptake data. Both compounds exhibited an induction period of about six minutes before oxygen uptake occurred under these experimental conditions. Uptake was linear with time. When the light source was turned off, oxygen uptake ceased; however, when the light was turned on again, oxygen uptake commenced immediately at the previous rate. The solutions became colored shortly after the light was turned on and darkened further upon continued exposure to light.

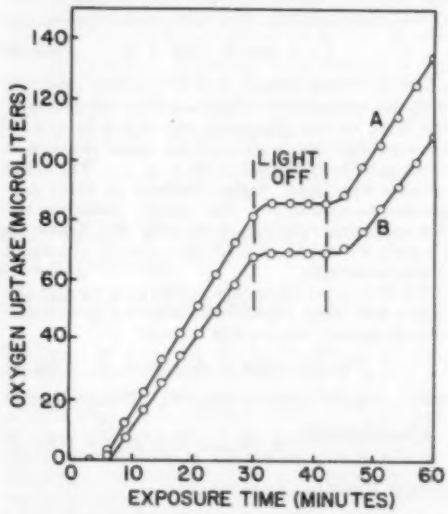

Fig. 1.—Plot of oxygen uptake data for (*A*) chlorpromazine hydrochloride, SKF, and (*B*) prochlorperazine ethanedisulfonate, SKF, unbuffered 5% solutions illustrating induction period, the linearity of the uptake with time, and the extreme light dependence of the oxidative degradation.

Other studies indicate that the rate of oxygen uptake and color formation of these solutions is affected by pH, the light spectrum and its intensity, oxygen tension, drug concentration, solvent, and the presence of certain additives. These observations have served as a prelude to some intensive studies of the factors influencing oxidative deterioration of phenothiazines and other oxidizable substances of pharmaceutical interest. Further results of these studies and their significance in pharmaceutical formulation and stability problems will be reported later.

* Received December 18, 1957, from the Research and Development Division of Smith Kline and French Laboratories, Philadelphia 1, Pa.

[1] Circular Warburg Respirometer (No. 5-130) with 15-ml. flasks, American Instrument Company.

[2] General Electric Sunlamp, Type RS, 115 volt-60 cycle, 275 watts.

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Book Notices

Naturally Occurring Quinones. By R. H. Thompson. Academic Press Inc., New York, and Butterworths Scientific Publications, London, 1957. vii + 302 pp. 14 x 21.5 cm. Price $9.

A comprehensive account of the quinone pigments is presented. The quinones are considered mainly from a chemical standpoint with emphasis on their reactions and constitution. The literature is covered to the end of 1956 and some references to papers published in 1957 are included. This book should be of particular interest to those concerned with natural or synthetic coloring materials.

British Pharmacopoeia 1958. Published under the direction of the General Medical Council. The Pharmaceutical Press, 17 Bloomsbury Square, London, W. C. 1, 1958. xxvi + 1012 pp. 15 x 22.5 cm. Price 3£, 3 s. plus postage. Distributed by The Rittenhouse Bookstore, 1706 Rittenhouse Sq., Philadelphia 3, Pa.

The British Pharmacopoeia 1958 is the ninth edition since the amalgamation of the London, Edinburgh, and Dublin pharmacopoeias in 1864. Since the 1953 edition, 160 new monographs, which include 60 new chemicals and 100 preparations of these substances, have been added. Among the 138 items not carried over from B.P. 1953 are aromatic spirit of ammonia, belladonna root, chenopodium oil, quassia, nutmeg, and the class of preparations named lamellae.

The antibiotic drugs have been increased by admission of 18 basic substances and their preparations; the corticosteroids have 12 new monographs, including prednisone and prednisolone; and radioisotopes of iodine and phosphorus are included. The newer anthelmintic piperazine derivatives are in and the old chenopodium oil is out. An odorless U.S.P. or N.F. drug might not be odorless by the new B.P. test which states: "Examine a sample of not more than 25 g. immediately after opening the package. If any odour is discernible, transfer the sample rapidly to an open container and re-examine after one minute. If the odour is still discernible, the sample does not comply with the description 'odourless.'"

Under "Indicators" (page 5), the operator is permitted to substitute indicators for one another providing they are in the same range of color change, "but where any doubt exists as to the equivalence of indicators for a particular purpose, the indicator specified in the text must be used."

The Introduction includes a list which shows major differences in titles of monographs in B.P. 1958 and the International Pharmacopoeia, 1st ed., vols. I and II. There are 27 appendixes. Appendix XXI is divided into 3 parts: A, disintegration test for tablets; B, modification of A for enteric-coated tablets; C, required diameters of uncoated forms are listed for official tablets, with the provision that "(a)where the diameter is given as ten thirty-seconds of an inch or more, the stated diameter may be exceeded by not more than one thirty-second of an inch. (b) a deviation of ±5 per cent is allowed." Permission is given in 11 out of the 120 monographs on tablets for the addition of a suitable coloring agent to the coating "because unfortunately uncolored coated tablets have not been made available. Similar provision has been made for some capsules." Where colored and uncolored coated tablets are available the colored tablet is not recognized as the B.P. article.

The B.P. 1958 is a technically excellent book of standards. The members of the British Pharmacopoeia Commission are to be commended on the performance of their duties in compiling this revision.

The Diagnosis and Treatment of Infection. By D. Geraint James. Charles C Thomas, Springfield, Ill., 1957. 14 x 22.5 cm. viii + 234 pp. Price $6.

This book aims to correlate diagnosis (aided by clinical laboratory findings) with treatment of infections (particularly with specific chemotherapeutic agents) for the student and practitioner of medicine. The text is divided into three main sections: Chemotherapeutic agents, Microorganisms causing human disease, and Infections of systems (respiratory, heart, etc.). Much useful information is compiled in 24 tabulations. A subject index is appended.

Pesticide Handbook. 9th ed. By D. E. H. Frear. College Science Publishers, State College, Pa., 1957. 216 pp. 15.5 x 23 cm. Paper bound $1.50; cloth bound $3.

A unique compilation of proprietary names of apparently all the pesticides used in this country, with the composition, manufacturer's name, and special applications. Besides other useful information, there is a classification of products by use of application (adjuvants, animal repellents, diluents, fungicides, etc.). This is an indispensable manual in the field of pest control.

Encyclopedia of Chemical Technology. 1st Supplement Vol. Edited by Raymond E. Kirk and Donald F. Othmer. Interscience Publishers, Inc., New York, 1957. xviii + 974 pp. 19.5 x 27 cm.

This first supplement volume of a useful compilation of documented information includes 51 articles ranging from (A) Acrolein, through (P) Psychopharmacological agents, to (W) Water demineralization. A 45-page subject index for this volume is appended. The composite index for the 15 numbered volumes of the Encyclopedia is included in vol. 15 which was reviewed in THIS JOURNAL, **46**, 390(1957).

The Terpenes. Vol. V. By the late Sir John Simonsen and W. C. J. Ross. Cambridge University Press, New York, 1957. ix + 662 pp. 14.5 x 22 cm. Price $15.50.

This volume, a continuation of Volume IV, covers the more complex triterpenes which occur in saps, resins, fish oils, and animal body fats. Hydroxy acids and lactones, hydroxyaldehydo, hydroxyketo acids, and the stereochemistry of the triterpenes are covered.

Emulsions: Theory and Practice. By Paul Becher. Reinhold Publishing Corporation, N. Y., 1957. ix + 382 pp. 15 x 23 cm. Price $12.50.

Modern developments in the theory and practice of emulsions are explained in this monograph. It begins with facts and theory of surface chemistry necessary for an understanding of the physical properties of emulsions as a function of emulsion composition. Next the theories of emulsion stability are covered. The chemistry of emulsifying agents, emulsifier efficiency, and the technique of emulsification are also discussed. The measurement of emulsion properties and commercially available emulsifying agents are covered in appendices. Most of the material in the book is based on developments of the last fifteen years, but earlier work of significance is cited.

Biochemical Preparations. Vol. 5. Edited by the 20-member editorial board with David Shemin as Editor-in-Chief. John Wiley and Sons, Inc., N. Y., 1957. ix + 115 pp. 15 x 23 cm. Price $4.75.

This volume follows the same general plan of presentation as the first four in the series. Isolation methods are particularly emphasized, but synthetic preparations are given for compounds of interest to biochemists, if the compounds are more readily obtained by syntheses. Methods for the preparation of twenty new compounds are described.

Accepted Dental Remedies 1958. 23rd ed. American Dental Association, Chicago, 1958. xiv + 214 pp. 15.5 x 20 cm. Price $3.

The material in this edition has been brought up-to-date and reorganized in order to make the book easier to use. One chapter provides a brief outline of the significance to the dentist of some of the drugs which physicians frequently administer to ambulatory patients who may then come to the dentist's office for concurrent dental care. The chapter on emergencies has been expanded and the desirability of recognizing the health status of the patient has been particularly emphasized. Of special pharmaceutical interest is the information concerning drugs of recognized value in dentistry, drugs of uncertain status more recently proposed for dental use, and some drugs once employed extensively, but now generally regarded as obsolete. Provisions of acceptance of products, a general index, a distributor's index, an index of current reports from the Council, and an index of more recent reports on products not listed are included.

Cahiers de Synthèses Organique, Méthodes et Tableaux d'Application. Vols. II and III. By JEAN MATHIEU and ANDRÉ ALLAIS with the collaboration of J. VALLS and P. POIRIER. Published under the direction of Léon Velluz. Maison & Cie, Paris, 1957. Vol. II, 266 pp. 15 x 22 cm. Price 6,000 fr. Vol. III, 322 pp. 15 x 22 cm. Price 4,000 fr.

Volume II of this series continues, under the general heading "Elaboration of structures," chapters: 3. Alkylation in aliphatic series; 4. Alkylation in aromatic series; 5. Arylation in aromatic series; and continues in Vol. III, chapters: 6. Alkyldienation; 7 and 8. Hydroxyalkylation and aminoalkylation in aliphatic series and aromatic series. A recapitulative table of functional groups and created arrangements, covering Vols. I–III is appended. The type, style, format, and presentation of material are the same for Vols. II and III as for Vol. I which was reviewed in THIS JOURNAL, **47**, 536(1958).

Scientific Russian. By James W. Perry. Interscience Publishers, Inc., New York, 1950. xxix + 816 pp. 15 x 23 cm. Price $7.50.

This textbook is designed for classes or for self study and its purpose is to teach a reading knowledge of scientific and technical Russian. It is divided into forty lessons most of which include a Russian reading exercise written to provide illustrations of those features of the language with which the text is concerned.

Molecular Structure and Organoleptic Quality. Society of Chemical Industry, 14 Belgrave Square, London, S. W. 1, and The MacMillan Co., New York, 1957. iv + 124 pp. 13.5 x 21.5 cm. Price $3.75.

This book includes eight papers read at a symposium held in Geneva, Switzerland, in May 1957, and sponsored by the Society of Chemical Industry. The titles and authors of the eight papers are: "Facts Old and New Concerning Relationships between Molecular Structure and Odour," by M. Stoll; "Physiology and Genetics of Organoleptic Perception," by H. Kalmus; "Tentatives de Mesure de L'Intensite de L'Odeur," by J. Sfiras et (Mlle) A. Demeilliers; "The Relationship between the Stereochemistry and Odorous Properties of Organic Substances," by Y.-R. Naves; "Structure and Odour," by M. G. J. Beets; "Odour and Molecular Vibration," by R. H. Wright; "Some Comments on Theories of Smell," by H. W. Thompson; and "Fundamentals of Odour Chemistry: A Summary," by L. Ruzicka.

The Neuroses and Their Treatment. Edited by Edward Podolsky. Philosophical Library, Inc., N. Y., 1957. xiv + 555 pp. 14 x 21 cm. Price $10.

This book is designed to present a comprehensive survey of the field by bringing together about forty representative papers containing the experience of specialists in handling all phases of the neuroses.

Subsidia Pharmaceutica I. Compiled and edited by the Scientific Center of the Swiss Pharmaceutical Society, and published by the Society, Zurich, 1957. 220 pp. 17 x 24 cm., ring binder, loose-leaf. Price 45 Swiss Francs.

This compilation of information, prepared especially for Swiss pharmacists, is divided into seven parts, the first written in French and the others in German. Part One consists of an alphabetic listing of international common names recommended by the World Health Organization for a large number of organic medicinals, together with the names for the same substances assigned by various pharmacopeias and other compendia (including U. S. P., N. F., and N. N. R.), their brand names, and also their structural formulas.

The second part deals with Therapeutic classes of medicinals and presently includes a general article on the Pharmacology of the autonomic nervous system and one reviewing Sympathomimetic agents; other articles will follow.

The five other parts consist of: (a) Methods of analysis of certain nonofficial medicinals; (b) A discussion of new galenical preparations (an excellent chapter on sulfonamide suppositories is included); (c) A commentary on "magistral prescriptions" (the chemical and physical properties of a number of nonofficial substances are described); (d) A description of new pharmaceutical apparatus (such as a small bacteriological filter, and an apparatus for distilling very pure water); (e) Some useful arithmetic formulas.

Additional information is to be supplied to subscribers in the form of loose-leaf page inserts. This means of providing up-to-date pharmaceutical information continuously is a unique and highly commendable undertaking of the Society, one for which much credit belongs to Drs. K. Steiger and J. Büchi, President and Editor, respectively, of the Editorial Commission responsible for the project.

Pharmacologic Principles of Medical Practice. 4th ed. By John C. Krantz, Jr. and C. Jelleff Carr. The Williams and Wilkins Co., Baltimore, 1958. xi + 1313 pp. 14.5 x 23 cm. Price $14.

The principal aim of the fourth edition of this treatise is the same as that established in the previous editions. Several extensive changes, however, have been made. The authors should be proud of their accomplishment in writing a thoroughly up-to-date textbook on pharmacology and therapeutics.

Surface Active Agents and Detergents. Vol. II. By Anthony M. Schwartz, James W. Perry, and Julian Berch. Interscience Publishers, Inc., New York, 1958. xv + 839 pp. 15 x 23 cm. Price $17.50.

Volume II supplements and extends the previous volume which was published in 1949. [THIS JOURNAL, **48**, 415(1949)]. It covers developments during the period 1947–1956 and includes processes for synthesizing and manufacturing surfactants, their special function and composition, recent advances in their physical and colloidal chemistry, and practical applications.

Atomic Energy in Medicine. By K. E. Halnan. Philosophical Library, New York, 1957. ix + 157 pp. 13.5 x 22 cm. Price $6.

A nontechnical introduction to an interesting new field of medicine. The most important uses of atomic energy in medicine are included.

Thrombelastography. By Pietro de Nicola. Charles C Thomas, Publisher, Springfield, Ill., 1957. x + 110 pp. 13.5 x 22 cm. Price $5.50.

The technical, experimental, and clinical aspects of thrombelastography are presented. It is intended as a supplement to the volume on the laboratory diagnosis of coagulation defects and includes a description of the apparatus and of the thrombelastographic technique.

Index of Plants of North Carolina with Reputed Medicinal Uses. By Marion Lee Jacobs and Henry M. Burlage. Published by Henry M. Burlage, University of Texas, Austin, 1958. xi × 322 pp. Price $7.50.

This work was prepared over a period of several years through the collaboration of the late Dean Jacobs of the University of North Carolina and Dean Burlage, presently Dean of the College of Pharmacy of the University of Texas. Not one index, as the modest title would have it, but seven indexes are gathered together here—indexes of scientific names, therapeutic indications, constituents, etc.; in addition to these there is the basic text which includes many data additional to those selected for indexing. This compilation represents an effective coverage of the book literature from the beginning up to 1940, and without question gives a more comprehensive survey of the North Carolina (and of the southeastern U. S.) medical flora than anything ever offered before between two covers. The fruit of long and detailed study, this book definitely merits a place in the library of any institution or individual interested in one of our great natural resources—botanical drugs. One thousand four hundred and forty-seven plant species are covered, representing either Indian or U. S. folk medicines or allopathic medicines of the present or recent past. Nomenclature, official status, range and habitat, constituents, parts used, uses, and preparations are given.

Neomycin, Its Nature and Practical Application. Edited by Selman A. Waksman. The Williams & Wilkins Company, Baltimore, 1958. x + 412 pp. 15.5 × 23.5 cm. Price $5.

This book is a comprehensive summary of the nature and utilization of neomycin, with an extensive bibliography of 691 references. The text is divided into four main sections: Microbiological and chemical aspects, Antibacterial and pharmacological properties, Clinical uses, and Miscellaneous uses. The 30 chapters represent the efforts of 33 contributors. Appended are a summary of specific clinical uses of neomycin, a list of neomycin preparations under therapeutic classifications, a list of manufacturers, and a general index.

The Extra Pharmacopoeia (*Martindale*). Vol. I. 24th ed. Published by direction of the Council of The Pharmaceutical Society of Great Britain. The Pharmaceutical Press, 17, Bloomsbury Square, London, W. C. 1, 1958. 12 × 18.5 cm. xxx + 1,695 pp. Price 3 pounds, 5s. (postage 3s).

This very valuable reference book has been revised to include material that has become available since the 23rd edition, which was reviewed in THIS JOURNAL, **42**, 266(1953). The same style and format have been retained, except that English titles of drugs are used throughout and, in the resetting, larger type has been used for abstracts and certain other information. The 24th edition requires about 1,700 pages compared with about 1,350 for the previous edition. The General Index, covering 100 pages, is a complete and helpful reference guide. The usefulness of The Extra Pharmacopoeia has been established for many years and it is recommended to pharmacists everywhere.

Elements of Mathematical Biology. By Alfred J. Lotka. Dover Publications, Inc., New York, 1958. xxx + 465 pp. 13.5 × 20.5 cm. Price $2.45.

This is a paper-back edition of the book first published as "Elements of Physical Biology." The 34 paragraphs of the text are divided under the four main headings: General principles, Kinetics, Statics, and Dynamics. References are given throughout the text. Author and subject indexes are appended.

Advances in Petroleum Chemistry and Refining. Vol I. Edited by Kenneth A. Kobe and John J. McKetta, Jr. Interscience Publishers, New York, 1958. xv + 641 pp. 15 × 23 cm. Price $13.50.

This is the first volume in a proposed series of annual compilations of progress reports. The petroleum and petrochemicals industry has been divided, for editorial purposes, into five main sections: Economics and future trends, Unit operations and design, Refining processes, Petrochemicals, and Mechanical equipment. Topics from these sections are included. An index is appended.

Einzeldarstellungen aus dem Gesamtgebiet der Biochemie. Neue Folge. II. Band "Die Biochemie der tierischen Gifte" von Erich Kaiser und Heribert Michl. Edited by O. Hoffmann-Ostenhof, Wien. Published by Franz Deuticke, Wien, 1958. VIII + 259 pp. 16.5 × 24 cm.

The first volume of this series dealt with the various factors which are involved in the blood clotting mechanism. Volume II is a critical survey and thorough treatise of the biochemistry of animal poisons. This is a very important field for chemists and particularly for biochemists. A comprehensive study of animal poisons from a biochemical approach has not been published before and this monograph is, therefore, of greatest interest. Other groups such as zoologists, pharmacologists, pathologists, and serologists have published papers and treatises on animal poisons, but never has it been done from a biochemical point of view.

The authors have performed an almost unsurmountable task by collecting data from about 10,000 publications. They present their findings in a clear way and supplement this information, when necessary, by their own experiments. The main achievement of this monograph is to present the first critical survey of an area which is equally important for biochemistry, pharmacy, pharmacology, serology, and many other related fields. The emphasis, however, is always on biochemistry.

This publication is well organized and well documented by literature references. Other monographs in this series are being prepared for publication. Volume III will be published shortly under the title: "Radioaktive Isotope in der Biochemie."

Le Livre Blanc. Formulaire Medical. Henri Perrier, 3 rue Aubriot, Paris 4e., 1958. 887 pp. 19.5 × 13.5 cm. Price Frs 1,425.

This 2,000-page book condenses in a convenient form a collection of information of considerable pharmaceutical and medical interest.

The information is presented in four different divisions. The first part is of particular interest to physicians, and presents résumés of classical treatments and the selection of specialties grouped according to their dominant active principle or their mode of action.

Part II includes a comprehensive list of pharmaceutical specialties in alphabetical order with information on their composition, properties, indications and contraindications, and the dosage forms available.

The third section includes a list of laboratories whose names are followed by useful commercial information and an enumeration of their specialties.

The fourth part presents information regarding illnesses which must be reported, vaccinations, a list of health resorts, and other information of particular interest to physicians and pharmacists in France.

The White Book, through the originality of its plan, its logical classifications, and the extent of its documentation is a useful reference book to practicing pharmacists and physicians not only in France, but in other countries. Its special value to pharmacists in countries other than France is that it enables them to locate information on drugs sold under proprietary names in Europe which is not ordinarily available from other sources.

Medical Electrical Equipment. Advisory Editor, Robert E. Molloy. Philosophical Library, Inc., New York, 1958. viii + 312 pp. 16 × 22.5 cm. Price $15.

This book supplies information on the principles, operation, and maintenance of medical electrical apparatus and devices, particularly the more technical equipment in hospitals, clinics, and surgeries. It describes the equipment, with illustrations of its component parts, and indicates the special care required to eliminate possible dangers to patients and staff. An index is appended.

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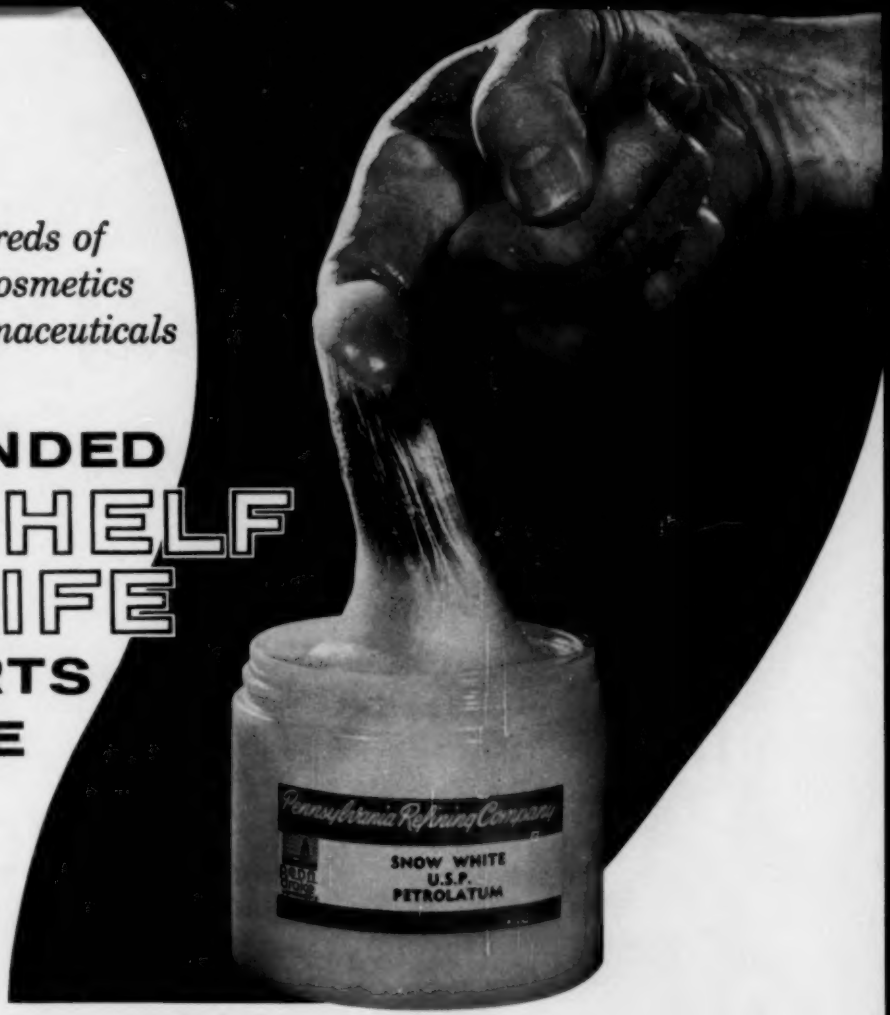

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